Approaches towards therapeutic development against chronic brucellosis in a mouse model

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Abstract

Brucellosis is the most common zoonotic disease worldwide. The intracellular localization of *Brucella* hinders the action of drugs that poorly cross cell membrane barriers. Additionally, when the immune response fails to clear the infection, chronic brucellosis ensues that becomes more challenging to treat with antibiotics. Therefore, two approaches, intracellular drug delivery and immunostimulation, have been explored in this dissertation, with an aim to develop a better therapeutic against *Brucella* infection in mice.

First, to overcome the cell membrane barriers, drug loaded nanoparticles were tested to treat *B. melitensis* infection in mice. Gentamicin loaded block-ionomer complexes (BICs) and magnetite block-ionomer complexes (MBICs) were tested *in vitro* and along with clusters of MBICs (MBIClusters) were tested *in vivo* as tools to deliver gentamicin intracellularly. While these complexes showed very high efficacy compared to free gentamicin against *Brucella* in macrophage cell culture, they failed to show similar efficacies in mice. Histopathological examination of kidneys from mice treated with MBICs or MBIClusters showed deposition of brown pigment-laden macrophages in peri-renal adipose tissue and the pigment was confirmed as MBICs or MBIClusters based on special staining

for iron. Additionally, it was found that doxycycline-gentamicin (DG) treatment results in better clearance of *Brucella* from infected mice compared to doxycycline alone.

Secondly, two vaccine candidates, irradiated *B. neotomae* (IBN) and outer membrane vesicles (OMVs), were tested as immunostimulants to treat chronic *B. melitensis* infection in mice in combination with antibiotics. The non-ionic block co-polymer Pluronic P85, when mixed with OMVs as an adjuvant showed significantly higher protection against *B. melitensis* challenge in vaccinated mice compared to those vaccinated with OMVs alone.

When tested as immunostimulants, there was no additive effect of vaccines and antibiotics on *Brucella* clearance from mice. However, IBN enhanced the production of IFN-γ while OMVs were associated with enhanced antibody production. This enhancement in the immune system resulted in the control of *Brucella* growth after the end of treatment. When given without antibiotics, vaccine alone failed to clear any *Brucella* from infected mice. The use of these vaccine candidates in combination with antibiotics shows a potential to prevent relapses in cases of brucellosis.

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List of Abbreviations

APCs	Antigen presenting cells
BICs	Block-ionomer Complexes
BSL3	Bio-safety level-3
CFUs	Colony forming units
CMI	Cell mediated immunity
ELISA	Enzyme linked immunosorbent assay
IFN-γ	Interferon gamma
IL-4	Interleukin 4
IL-10	Interleukin 10
IL-12	Interleukin 12
i.p.	Intraperitoneal
i.v.	Intravenous
i.m.	Intramuscular
LD_{50}	Lethal $dose_{50}$
LPS	Lipopolysaccharide
MΦ	Macrophage
MBICs	Magnetite Block-ionomer Complexes
MHC-I	Major histo-compatibility complex I
MHC-II	Major histo-compatibility complex II
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium)
NK	Natural killer cells

OMVs	Outer membrane vesicles
PBS	Phosphate buffer saline
PAA	Poly(acrylic acid)
PEO	Poly(ethylene oxide)
PPO	Poly(propylene oxide)
RPM	Revolutions per minute
SEM	Standard error of the mean
Th1	T Helper cell 1
Th2	T Helper cell 2
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha

General Introduction

Most chronic infections like *Mycobacterium* and *Brucella* are difficult to treat and have high treatment failure and relapse rates (53, 62). Brucellosis is the most common zoonotic disease worldwide and is associated with heavy economic burden (33). There is no recommended treatment for animals as it is not economically viable. Recommended treatment for human brucellosis includes a combination antibiotic therapy for 6-8 weeks (169). Moreover, treatment of complicated brucellosis requires up to two years of antibiotic treatment along with surgical intervention (4). Even after following a complete antibiotic regimen to treat brucellosis, the treatment failure and relapse rates are 1-5% and 5-10% respectively (169).

Drugs that are very effective against *Brucella* in culture medium fail to clear infection, due to several reasons including inability to cross cell membrane barriers, rapid elimination from the body, inactivation of drug at low pH and inability to attain therapeutic concentrations (166). Aminoglycosides like gentamicin are very effective in culture medium against *Brucella* but fail to cross cell membranes to kill intracellular *Brucella* (38). Drug carrying nanoparticles have been shown to overcome this issue to deliver drug intracellularly (46, 106). Similar to *Mycobacterium* infection, the host immune system fails to clear *Brucella,* leading to chronic and complicated brucellosis. Stimulating the immune systems of infected hosts could be a way to eliminate hiding *Brucella* and improve therapy (4).

The primary focus of this dissertation is to develop a better treatment for chronic *B. melitensis* infection in a mouse model. Two approaches to achieve this goal include: 1) improving drug delivery to the *Brucella* infected cells by using gentamicin loaded nanoparticles 2) testing the efficacy of vaccines as immunostimulants, in combination with conventional antibiotics to treat chronic *Brucella* infection in mice.

Comparative efficacy of free gentamicin and gentamicin loaded in nanoparticles, use of adjuvants to enhance the efficacy of outer membrane vesicles and combined effect of vaccines and antibiotics on the treatment of chronic *B. melitensis* infection in mice are described herein.

Chapter 1: Literature review

Brucellosis treatment and vaccines: Current Scenario

Brucellosis and *Brucella*

Brucellosis is one of the most ancient diseases and has been persistent for thousands of years as evident by the discovery of typical lesions in human skeletons dating back from 79 AD (26). Sir David Bruce isolated the organism, *Brucella melitensis* in 1886 from the spleens of dead British soldiers stationed on the island of Malta (25). He traced the source of infection to goat's milk. The disease was named as Malta fever and the identified organism was called as *Micrococcus melitensis* (Roman name of malta 'Melita'). In 1897 Bernhard Bang identified the causative agent for contagious abortions in cattle and named it *Bacillus abortus* and the disease as Bang disease (14). Finally, in 1917 the two organisms were found to be identical and named '*Brucella'* in honor of Sir David Bruce.

More than a century after its discovery, brucellosis is still the most common zoonotic disease worldwide (33). Almost all domestic and some wild animals are affected by different species of *Brucella*. Worldwide, more than 500,000 new human brucellosis cases appear every year (131). The disease is one of the most commonly acquired laboratory infections and is an occupational hazard for the veterinarians (63). *Brucella* has a very low infectious dose for humans (10-100 bacteria) and can be easily aerosolized thus, it is attractive as an agent of bioterrorism (132). Classified as Category B agent by Centers for Disease

Control (CDC) (135) and National Institute of Allergy and Infectious Diseases (NIAID) (186), *Brucella* has been included on the list of potential bio-weapons by the World Health Organization (WHO), the North Atlantic Treaty Organization (NATO) and the Biological and Toxin Weapons Convention (BTWC) (132). Almost all the major national programs for offensive biological weapons development have used *Brucella* (156). Japan experimented with *Brucella* in 731 Manchuria Unit (a covert biological and chemical warfare research and development unit of the Imperial Japanese Army) during World War II. *B. suis* was first weaponised by USA in 1952 and field tested (28). In the former Soviet Union, *Brucella* was developed by the Biopreparat (Soviet biological weapon program) for offensive purposes and antibiotic resistant strains were used and weaponised both in dry and liquid forms (132).

Brucella is a Gram-negative bacterium, belonging to the α-2 subclass of protobacteria (119) and is genetically related to *Ochrobactrum spp., Agrobacterium spp., Rhizobium spp.* and *Phylobacterium spp* (185). The bacterium is coccobacilliary in shape and is 0.5-0.7μm in diameter and 0.6-1.5μm in length. *Brucella* is non-motile and non-spore forming organism. It is believed that originally *Brucella* was a plant pathogen but during evolution jumped to mammalian hosts (118, 154). Six major species of *Brucella* are known that are classified on the basis of pathogenicity and host preference, *B. melitensis* (sheep), *B. abortus* (cattle), *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (goats) and *B. neotomae* (desert wood rats). Two new species have been isolated from marine mammals, *B. ceti* (whales, dolphins and porpoises) and *B. pinnipedialis* (seals and walruses) (43, 150). Recently, one additional species, *B.*

microti has been identified in voles (158) and another one, *B. inopinata* isolated from breast implants (159). All these species show a high interspecies DNA homology in DNA-DNA hybridization assays but are very specific for the host they infect (187, 188). The major pathogenic species to humans are *B. melitensis, B. abortus* and *B. suis* (33). *Brucella* spp. contains two chromosomes, 2.1 Mb and 1.15 Mb and contains no native plasmids. Just like all other Gram negative bacteria, *Brucella* contains lipopolysaccharide (LPS), the presence or absence of which decides whether the species is designated as "smooth" or "rough" (155). *B. canis* and *B. ovis* are naturally occurring rough *Brucella* species and do not cause infection in humans.

Brucellosis is endemic in Mediterranean regions, Middle East, Latin America, and parts of Asia (143, 168). The situation is improving in European countries but the disease is still endemic in Greece, Spain, Portugal and southern Italy. Brucellosis is emerging as a serious concern in Kazakhstan, Kyrgyzstan and Mongolia (132). The disease has been eradicated in most developed countries but is found in humans as a result of international travel and consumption of contaminated food products (114). The best example is the USA, where disease constantly travels from neighboring Mexico through food products and travel (36). North Africa is endemic with brucellosis while the situation in sub-Saharan Africa has not been thoroughly evaluated (132). Despite being endemic in many countries, the disease is still poorly diagnosed both in humans and animals. Major reason for this is the slow pathogenesis induced by the bacterium and confusing symptoms of the disease that resemble those of many other diseases and make diagnosis difficult.

Brucella pathogenesis

Transmission of *Brucella* to humans mostly occurs due to the consumption of contaminated milk and cheese (66). It could also happen as an occupational exposure to infected animals via their carcasses, uterine secretions or aborted fetuses. The symptoms of brucellosis in humans can vary from mild, like fever, fatigue, myalgia (153) to organ specific like splenomegaly, hepatomegaly and lymphadenopathy (132) to complicated situations such as arthritis, spondylitis, meningitis, and endocarditis (165, 171). As human brucellosis is directly dependent on animal infections, control of the latter is very important. *Brucella* infection in bovines and swine principally leads to mass abortions during the last trimester of pregnancy (27). The main symptoms in males are orchitis, epididymitis, vesculitis and infertility. Abortions are uncommon in canine and ovine brucellosis cases but infertility in males frequently occurs (23, 192).

Brucella is a facultative intracellular pathogen. It infects both professional phagocytes like macrophages and non-professional phagocytes, such as trophoblasts. *Brucella* does not contain classical virulence factors like exotoxins, endotoxins, cytolysins, plasmids, fimbria etc, but has specific virulence factors that are required for invasion and intracellular survival (162). The course of *Brucella* infection can be divided into three phases: the onset of the infection when *Brucella* invades the host and the first clinical signs are evident, followed by the acute phase of infection during which *Brucella* multiplies rapidly in the targeted organs (spleen and liver) and lastly, the chronic phase of infection. This phase has a plateau phase when the number of *Brucella* in targeted organs

remains constant, and declining chronic phase during which the number of *Brucella* decreases and is associated with inflammation (108).

Brucella evades the host protection mechanisms and survives inside the host cells. Compared to *Salmonella*, *Brucella* has fewer negative charges on its surface that minimizes binding of complement, microbicidal defensins, bactenecins, cathelicidins and other bactericidal cationic molecules (56). Also, *Brucella* LPS induces very low immune responses in terms of induction of pro-inflammatory cytokines (64). Smooth unopsonized *Brucella* enters macrophages through cholesterol-rich lipid rafts. Rough strains of *Brucella* have decreased ability to survive intracellularly, illustrating the importance of LPS O-side chain (137). During internalization *Brucella* relies on its two-component system, BvrR/BvrS that regulates the expression of *Brucella* outer membrane proteins (OMPs) during invasion (67). Once inside the macrophage, *Brucella* cyclic-β glucans (components of outer membrane) prevent phagosomal-lysosomal fusion (9). However, some of the *Brucella* are susceptible to killing via reactive oxygen intermediates and radicals (e.g. superoxide anion, H_2O_2 etc) when *Brucella* containing phagosomes fuse with lysosomes. Cu/Zn superoxide dismutase (SOD) is produced by *Brucella* and helps to protect against the reactive oxygen species. *Brucella* deletion mutants in *sodC* cannot prevent the killing as efficiently as the wild type strains (60). *Brucella* is not a strong inducer of nitric oxide in macrophages (191). The acidic environment of endosomes helps trigger expression of *Brucella* genes such as *virB* genes that encode components of the type IV secretion system, further helping the development and migration of the *Brucella* containing vacuole (BCV) closer to the rough endoplasmic reticulum

(RER) (24) (Figure 1.1). The BCV acquires membranes and nutrition from the RER and becomes a niche for *Brucella* survival and multiplication. Infected macrophages localize in the organs of reticuloendothelial system, such as spleen and liver, leading to chronic infections. Also, *Brucella* prevents the apoptosis of macrophages and survives and replicates within infected phagocytes (65). This protection against apoptosis has been correlated with the up-regulation of *Brucella's* A1 gene, a homologue of Bcl-2 family of anti-apoptotic genes. *Brucella* infected cells are also more resistant to Fas-ligand or IFN-γ induced apoptosis.

During pregnancy in infected ruminants, *Brucella* migrates to placental trophoblasts and fetal tissues. There are several factors that support extensive multiplication of *Brucella* at this site: 1) availability of erythritol, a four carbon sugar that is preferred by *Brucella* as a carbon source over glucose, 2) higher concentration of iron in trophoblasts as they are erythrophagocytic and *Brucella* needs iron for erythritol metabolism, and 3) lower immunity at the site to prevent fetal rejection (147). Rapid multiplication of *Brucella* inside placental trophoblasts disrupts the placenta and leads to abortion (27). The aborted fetus and the tissues then become the source of infection for other animals. The ability to escape the immune system and colonize host cells is the key to *Brucella* infection in almost all domestic species of animals, wild life animals, and humans.

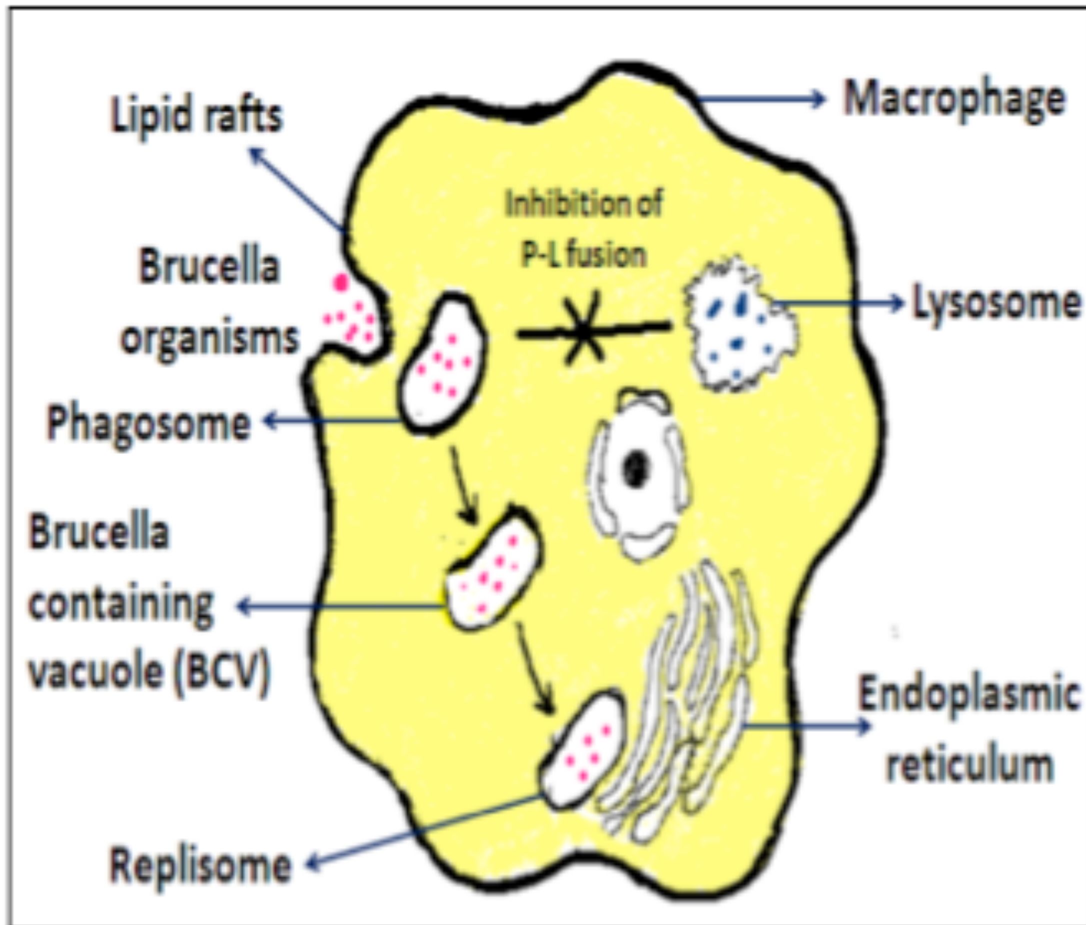

Figure 1. 1: Entry and survival of *Brucella* inside macrophages.

Brucella enters inside the host cells via lipid rafts, prevents phagosomal-lysosomal fusion and then migrates to specialized compartments called *'Brucella* Containing Vacuoles' (BCV) in vicinity of endoplasmic reticulum and survives and replicates inside replisomes**.**

Host Immune responses against *Brucella* infection

The mammalian immune system, characterized by innate and adaptive immunity, works against invading pathogens. The innate immune responses are non-specific and lack memory, while the adaptive immune responses are antigen-specific and retain memory. Innate immunity includes polymorphonuclear cells (PMNs) and non-activated macrophages that circulate in the body and eliminate pathogens by non-specific killing (130). Further, the complement system helps in elimination of pathogens by the deposition of proteins on bacterial surface and which acts as opsonins to attract phagocytic cells. As mentioned before, compared to other intracellular pathogens, *Brucella* resists complement binding on its surface (49, 95). The classic complement pathway and the lectin pathway are shown to cause some destruction to invading *Brucella* in mammalian host but the alternative complement pathway is not effective.

Pathogen associated molecular patterns (PAMPs) like LPS, lipotechoic acids, lipoproteins, ornithine containing lipids, and flagelin are recognized by toll like receptors (TLRs) of the innate immune system (85) and result in production of IL-12 and the recruitment of natural killer (NK) cells. *Brucella* PAMPs are low activators of the innate immune system. *Brucella* prevents recognition by TLR-4 by modifying its lipid A moiety of LPS and down-regulates inflammatory responses (73, 99). After engulfing *Brucella*, monocytes, macrophages or dendritic cells (DCs) produce very low amounts of IL-12 and TNF-α that further reducing immune stimulation.

In general, adaptive immune responses against intracellular pathogens require strong cell-mediated immunity (CMI). The major components of CMI are type I proinflammatory cytokines, IFN-γ produced by CD4+ and CD8+ T cells, cytolytic activity by CD8+ T cells and NK cells, and opsonization by IgG2a antibodies (Figure 1.2). IFN-γ has several roles in fighting against infections such as, upregulation of MHCI and MHCII molecules on cell surfaces inducing higher antigen recognition and presentation. This in turn increases the pinocytosis and phagocytic activity of macrophages, increases production of nitric oxide, lysosomal enzymes, reactive nitrogen species and oxidative burst mechanisms in phagocytic cells that result in higher bactericidal activity (152, 160).

Brucella interferes with different immune mechanisms that hinder the host's ability to clear *Brucella* infection. Both IL-12 and TNF-α have been shown to be important for the control of *Brucella* infection in a mouse model (195, 196). Depletion of either cytokine has been shown to result in decreased control of *Brucella,* as they are the key mediators from innate to adaptive immune response and for the production of IFN-γ. Another cytokine IL-10, an anti-inflammatory cytokine thst affects both production and cell response to IL-12, is produced as the result of interaction between *Brucella* and TLRs on macrophages (61). This further decreases the production of IFN-γ and thus control of the disease. IFN-γ is the key component for the control of *Brucella* infection. IFN-γ deficient mice die following a *Brucella* infection. Both CD4+ and CD8+ T cells produce IFN-γ in *Brucella* infected mice. However once inside the host cells, as a result of deposition of LPS on host cell membrane, *Brucella* interferes with MHC II presentation and thus with CD4+ T cells (51). This results

in reduced production of IFN-γ. Several studies have shown that *Brucella* infections do not induce the secretion of IL-4, a major Th2 cytokine (12, 13, 134).

Humoral immune responses characterized by B-lymphocytes and antibody production by plasma cells has been shown to provide marginal protection against *Brucella* infection. This involvement depends largely on the animal species infected (8, 104, 148). In humans *Brucella*-specific IgM antibodies against LPS appear during the first week of infection followed by IgG in the second week. Titers of both IgM and IgG persist until one year following the infection but have very little role in the overall host immune response (131). In mouse models, antibodies against *Brucella* LPS have been shown to provide protection against *B. melitensis* (30, 103, 117). Although, many aspects are still not fully understood, knowledge regarding host-*Brucella* interaction and host immune responses against *Brucella* has been very important for the development of vaccines and drug targets.

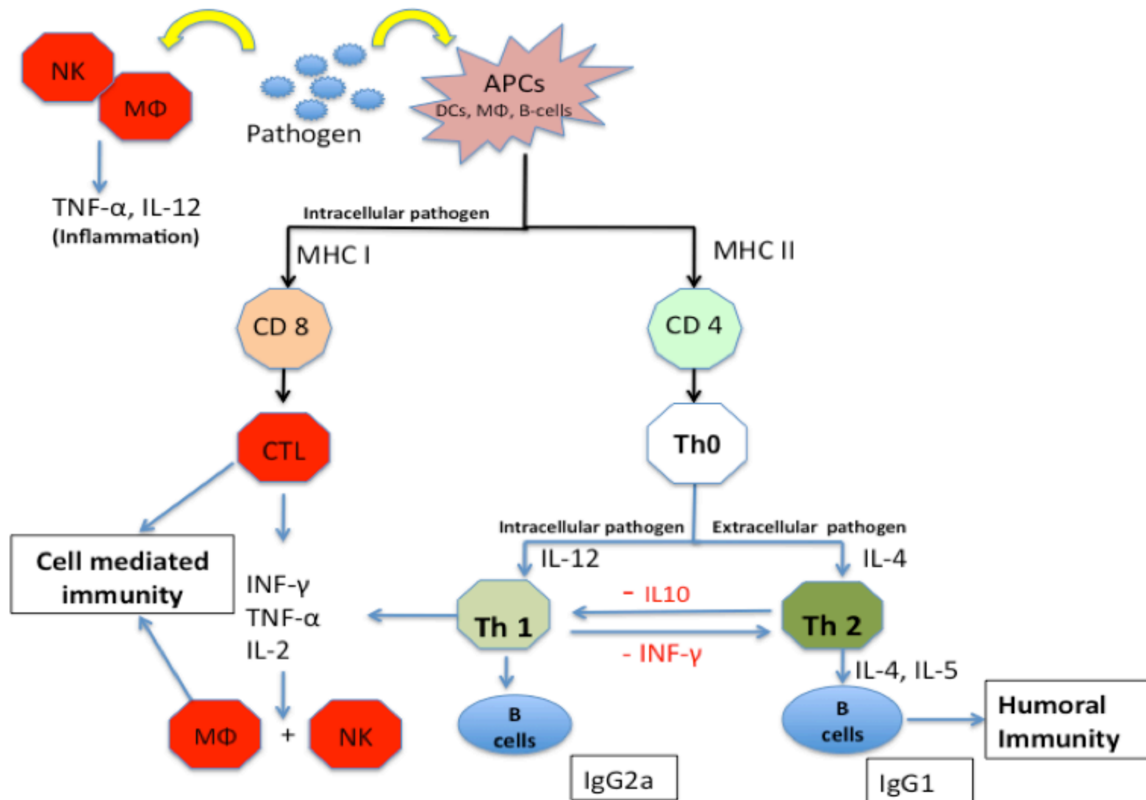

Figure 1. 2: General immune response against pathogens.

Depending on whether the pathogen is extracellular or intracellular, different arms of the immune system, i.e. Th1 and Th2, get activated to help eliminate the pathogen. Antigen presenting cells (APCs), like macrophages, dendritic cells and B cells, destroy the pathogens and present short peptides sequences in association with either MHC-I or MHC-II molecules. MHC-I molecules present endogenous antigens to CD8+ cells as a result of which cytotoxic T cells (CTLs) are generated. MHC-II presents peptides to naïve Th0 cells, which are further differentiated into Th1 (secretes IFN-γ) and Th2 (secretes IL-4) cells. CTL and Th1 cells together lead to a cell-mediated immune response while Th2 cells lead to a humoral immune response.

Brucellosis Vaccines

Even though brucellosis is the most common zoonotic disease worldwide, there is no human vaccine available. As described previously, cell-mediated immune responses are more important than the antibody response in eliminating *Brucella* in a host. Thus, any potential vaccine should induce predominantly Th1 and cytotoxic responses to mimic the natural *Brucella* infection. Different types of vaccines like live attenuated (smooth and rough strains), subunit and DNA vaccines have been demonstrated to protect against different species of *Brucella* in laboratory models. However, only a few could be used in the field due to inefficacy, safety, cost of production, stability and storage (161).

The first vaccine for use in cattle was attenuated smooth *B. abortus* strain 19 (7). This live attenuated vaccine was developed accidently when the cultures were left on the laboratory bench for several years and then found to be attenuated in mice and cattle and provide protection against bovine brucellosis (105, 176). The mechanism of attenuation is still not understood, but is related strain's inability to metabolize erythritol (44). The vaccine is widely used outside the U.S.A. to prevent bovine brucellosis, but it is fairly virulent to humans (52). Additionally, the presence of O-side chain in the strain leads to the production of O-side chain (LPS) antibodies that make it difficult to differentiate vaccinated vs. infected animals (116). Other moderately successful smooth-strain based vaccines have been used. H38 is a formalin killed *B. melitensis*, which is protective, but is associated with local reactions at the site of inoculation (161, 190). *B. suis* strain 2, developed in China, was found to be protective in sheep and

swine against *Brucella* challenge, but antibody titers were found to decline after 1 year post vaccination (22, 121). Although live attenuated smooth strains of *Brucella* provide protection, they cannot be used to satisfy “test and slaughter” regulations, as it is not possible to differentiate between vaccinated and infected animals. Thus, a rough mutant would be the best option if it provides protection against *Brucella* infection without interfering with disease surveillance.

B. abortus strain 45/20 is a rough, live attenuated strain used to prevent bovine brucellosis but it was found to revert to smooth form after administration (29, 142). In the United States the S19 vaccine was replaced by rough attenuated strain RB51 in 1996, which particularly helped during the later stages of the brucellosis eradication program (reviewed in (161)). Strain RB51 does not elicit an antibody response against smooth LPS, thus does not interfere with the diagnosis of naturally infected animals (104, 126).

The live attenuated smooth *B. melitensis* strain Rev-1 is very effective in small ruminants (3, 20). Strain Rev-1 is widely used in countries like Mexico but is still not approved in the USA because it can cause abortions in pregnant animals (21). Strain Rev-1 was reported to be shed in milk and was considered as a reason for the human infections in Israel and Middle East (15). To date, there is no approved vaccine for swine and canines.

<u>*Brucella neotomae* as a vaccine candidate:</u> *B. neotomae* was first isolated in 1957 from desert wood rats in the western United States (178). So far its potential to cause disease in humans or other animal species is not known. The use of *B. neotomae* as a

vaccine against *Brucella* infection was first described when it was shown to control swine Brucellosis (177). Recently, it has been shown that irradiated *B. neotomae* that loses its ability to replicate but remains metabolically active, and provides protection against *B. melitensis, B. suis* and *B. abortus* in mice (120). As *B. neotomae* is considered as non-pathogenic, it presents a great opportunity for testing as a human vaccine against *Brucella* infection.

<u>Outer membrane vesicles (OMVs) as a subunit vaccine:</u> First identified in *Psuedomonas aeruginosa*, OMVs are double membrane structures released by Gram negative bacteria during all phases of growth both *in vitro* and *in vivo* (111). Originally called as "Blebs", OMVs are mainly comprised of outer membrane proteins, lipopolysaccharide and periplasmic proteins (58, 72, 75). OMVs are heterogeneous spheroid shaped vesicles with size ranging between 10-300 nm in diameter (19). OMVs are not the product of cell death but their production increases at times of stress (112, 197). Moreover, it has been shown that vesiculation is an important natural phenomenon to endure stress in Gram-negative bacteria. Vesiculation mutants of *E. coli* show the reduced survival on exposure to envelop stressing mechanisms compared to wild type *E. coli* while over-producing strains survived better than wild type (113). Antibiotic treatment has been shown to enhance the production of OMVs by Gram-negative bacteria but the composition is different than the naturally shed OMVs (39, 88).

OMVs serve different purposes in different bacterial species, such as exporting factors related to virulence, antimicrobial resistance, interbacterial communication and immunomodulation (reviewed in (97)). In the case of *Legionella spp*. it has been shown

that OMVs prevent phagosomal-lysosomal fusion in infected cells and help in remodeling the bacterial surface to the intracellular replicative form (48). Vesiculating bacteria show better survival in mixed populations of bacteria by secreting periplasmic peptidoglycan hydrolases packaged in OMVs that kill other co-cultured bacteria (102). OMVs are shown to be involved in biofilm formation and colonization (80, 89) and as quorum sensing (109). OMVs also function as the delivery vehicle to transport bacterial toxins and other virulence factors to the host cells. For example, enterotoxigenic *E. coli* produces a heat labile enterotoxin and delivers it to epithelial cells via OMVs (75, 91). In some cases, OMV-associated toxins are found in the biopsy of infected tissue as well. For example, OMVs containing VacA toxin (toxin secreted by *Helicobacter pylori*) were found in the human gastric epithelium biopsy samples from *H. pylori* infected individuals (50). Toxins associated with OMVs are usually protease resistant and facilitate host-pathogen interactions (93). Other non-toxin virulence factors associated with OMVs include adhesins (93), proteases (149), procoagulants and fibrinolytic factors (157).

OMVs are significant activators of the host immune system and thus present an option to be used as acellular (subunit) vaccines against the bacterial infections. The most abundant molecule present in OMVs that is responsible for immune stimulation is LPS (41). LPS present in Gram-negative bacteria interacts with TLR-4 receptors and triggers a proinflammatory response and septic shock (129). However, LPS present in OMVs differ greatly from purified LPS with respect to its potential to stimulate innate immunity, distribution in host and clearance from host. LPS, along with other components of OMVs, elicits a more pathogen-specific immuno-stimulation compared to purified LPS or purified proteins (41). Other major molecules that generate a strong proinflammatory

response by OMVs include porins and adhesins (57, 180, 193). OMVs are also capable of stimulating the adaptive memory response as well. *Salmonella* OMVs are shown to activate macrophages and dendritic cells, increase MHCII expression, production of TNF-α and IL-12 and activate CD4+ T cells (5). This indicates that proteins in the OMVs can be effectively processed and presented by APCs and can trigger downstream immune responses. OMVs from *H. pylori* and *P. aeuroginosa* have been shown to induce IL-8 secretion from epithelial cells (17, 84).

The most successful effort to use OMVs as a vaccine so far is the commercial vaccine for human use to protect against *Neisseria meningitides* (reviewed in (74). These OMVs have been shown to trigger the production of numerous proinflammatory cytokines like TNF-α, IL-1 and IL-8 from neutrophils (100). Several different formulations of the OMV vaccines used depend on the strain in a particular geographical area. All preparations have been shown to stimulate protective mucosal and systemic bactericidal antibodies (55). The presence of LPS has been shown to be important in *Neisseria* OMVs. OMVs obtained from LPS deletion mutants of *Neisseria* are poor inducers of immune responses (37, 54). Thus, LPS acts as a natural adjuvant in OMVs and it has been shown that a mixture of LPS and outer membrane proteins in OMVs is required to generate effective host immune responses. All the commercial preparations of *Neisseria* OMVs are manufactured from detergent treated bacteria that help in reducing the endotoxic effect of OMVs without completely eliminating LPS.

Recently, we have shown the efficacy of *Brucella* OMVs as vaccine to protect against *B. melitensis* challenge in mouse model (11). The protection provided was equivalent to the protection provided by commercial Rev1 vaccine. Additionally, protein analysis of

Brucella OMVs revealed the presence of *Brucella* protective antigens like superoxide dismutase (127, 128), outer membrane protein 31 (omp31) and omp25 (40, 42) in OMVs.

Like any other subunit vaccine OMVs also require a strong adjuvant to elicit a higher and more specific immune response. Adjuvants cannot only guide the immune response towards either Th1 or Th2 pathways but can also help to improve the immune responses generated by a subunit vaccine. A strong adjuvant may be able to replace the need for the presence of LPS in the OMVs, thereby eliminating the risk of endotoxicity associated with it.

Adjuvants for subunit vaccines

First discovered almost a century ago, an adjuvant (derived from Latin, adjuvare = to help) is a substance which, when added to a vaccine, enhances the immune response elicited by the administered antigen (34). Adjuvants are of much greater importance when using recombinant proteins or peptides as vaccines. Highly refined antigens focus only on protective targets without inducing unwanted reactogenicity, but at the same time they induce a poor immune response, thus the role of adjuvants becomes important (31). A lot of natural or synthetic substances have been reported to have adjuvant properties but due to safety concerns only few are licensed for human use (184). In United States, only Alum based adjuvants are approved for human use (18), while in other countries including European Union, some oil/water emulsion based adjuvants are also approved (101, 189).

Different types of adjuvants have been shown to be effective but the mechanism of action is mostly unknown. An adjuvant may act in one or more of five ways: immunomodulation, presentation, induction of CD8+ cytotoxic T cells, targeting, or depot generation (reviewed in (34)). Adjuvants that act as immunomodulators, push the immune system either towards a Th1 or Th2 response by modifying the cytokine network. The most common examples include aluminum salts, cytokines and bacterial endotoxins that induce Th2 and Th1 respectively (87, 107, 181). Aluminum salts are the only adjuvants approved for human use in the United States but multiple doses are needed to attain the desirable effect.

As depicted in Figure 1.2, the Th1/Th2 switch of the immune system is determined at the stage where antigens are presented by MHCII histocompatability complex, leading to clonal expansion of Th0 cells. Presentation of antigenic epitopes decides its interaction with APCs and the immune response against antigen at this point (122). Some adjuvants interact with antigens in such a way that conformational epitopes are more effectively presented to the immune cells, leading to a stronger immune reaction. Common examples include water-in-oil emulsions like incomplete Freund's adjuvant (IFA). IFA was found to be too reactogenic in humans and its use have been discontinued. Later, refinement of the base materials resulted in new versions of water-in-oil emulsions, these are acceptable for use in therapeutic vaccines like Montanide emulsions which are used in ongoing vaccine trials against several cancer types. On the other hand, oil-in-water emulsions that also include non-ionic block copolymers have been shown to act by the induction of local inflammation and increased recruitment and

activation of APCs (78). This leads to enhanced antigen presentation and targeting of antigen to APCs as well.

For the induction of CTL responses, an antigen needs to be processed in the cytosol of APCs and presented by MHC I complexes (Figure 1.2). Adjuvants capable of interacting with the cells membranes such that the antigens associated with it will be deposited in the cytosol of the APCs are effective in elucidating the higher CTL responses. These adjuvants are of great importance for vaccines against intracellular pathogens. Certain cytokines like IL-2, when used as an adjuvant, have been shown to enhance the CTL induction (47). Targeting of the antigens to specific targets like APCs is highly desirable as most antigens are degraded in serum by proteases or eliminated by the liver (2, 86). This targeting can be achieved by adjuvants that have moieties for the cell surface molecule recognition like recognition of GM-1 gangliosides on macrophages (146). Another effective mechanism of action of adjuvants is by creating depots that lead to continuous release of the antigen and prevention of clearance by the liver. Short-term depots include aluminum salts and water-in-oil immulsions while long term depots includes the use of synthetic polymers like polylactide coglycolide (PLGA) that produce microspheres/nanospheres encapsulating antigens (34). Most adjuvants work through multiple mechanisms the details of which are still not very clear.

Pluronics or Poloxamers are co-polymers of hydrophilic blocks of polyethylene oxide (PEO) and hydrophobic blocks of polypropylene oxide (PPO). Non-ionic block copolymers are used commercially in shampoos, mouthwashes, cosmetics, topical ointments and contact lens solution and are considered safe. The idea of testing

pluronics as adjuvants started with the observation that Pluronic L121 and similar molecules are amphipathic surface-active agents and were able to bind to proteins and other antigens. This suggested that because of their surface properties the Pluronics would enhance the interaction between antigens and antigen presenting cells (79). Since then several Pluronics and related molecules have been shown to act as adjuvants and enhance the cytokines and antibodies formed in response to a variety of antigens following subcutaneous administration. The adjuvant activity of Pluronics has been shown to be dependent on the percentage of PEO in the polymer. It has been shown that copolymers with 10% PEO successfully augment Type II T lymphocyte responses while ones with lesser concentrations of PEO increases both Type I and II responses (124). Pluronics with more hydrophobic entities disrupt the cell membranes associated with the plasma membrane or that of phagosomes or endosomes and channel antigens towards presentation via MHC class I in macrophages and other antigen presenting cells. Hydrophilic Pluronics enhance the presentation of antigens via MHCII molecules in APCs (77). However, Pluronic CRL-1072 was shown to stimulate the production of IL-8, TNF-alpha and GM-CSF by macrophages in a dose dependent manner (90). Polaxamers 188, 407 and 908 have been shown to stimulate the phagocytic activity of human neutrophils and murine macrophages (81, 115). Thus, Pluronics could be used as adjuvants but are restricted because of some toxicity issues. Recently, Pluronic P85, which is amphiphilic in nature, has been shown to be less toxic than hydrophobic Pluronics. Also, it has been shown that P85 can be a biological response modifier and can be used for the treatment of multi-drug resistant cancers. When P85 was injected in mice along with DNA, it acted as a vaccine adjuvant as it

enhanced the migration and maturation of APCs to the site of injection (59). This further enhanced the expression of the transgene carried by APCs in distant immune organs In one study, when administered before the challenge P85 alone was able to provide protection against a *Listeria monocytogenes* challenge in a mouse model (123). Based on the *in-vitro* findings where incubation with P85 resulted in depletion of ATP in Caco-2 cell cultures, it was suggested that depletion of ATP by P85 translated to its ability to provide protection. Other speculations included the ability of P85 to modify cytoplasmic vesicles and stimulation of innate immune response in mice but this has not been proven.

Thus, Pluronic P85 could be used as an adjuvant for an acellular or subunit vaccine. Because of its ability to modify biological systems, it might have an impact above that of an inert carrier that could be useful in enhancing the protection provided by vaccines.

Treatment of Brucellosis

Conventional therapeutics: Treatment of animal brucellosis, particularly in large animals, is not economical thus there is no recommended regimen. Historically the mortality rate associated with brucellosis in human is less than 2% (95). Combination therapy has been found to be more effective with lower relapse rates than monotherapy (4). A relapse of brucellosis is characterized by reappearance of signs and symptoms of the disease and new positive blood cultures within 12 months post-therapy while a therapeutic failure is the persistence of signs and symptoms at the end of the treatment (170). The WHO recommended regimen for the treatment of human brucellosis include

tetracycline (e.g. doxycycline) in combination with rifampicin for 6 weeks. Rifampicin could be replaced with streptomycin (i.m. 2 weeks) (1). In different human trials, it was found that DS (doxycycline-streptomycin) was associated with fewer relapse rates than DR (doxycycline-rifampicin) (10, 172, 173). But the WHO recommendations still incude DR because of the risk associated with injecting streptomycin in developing countries (4). As the choice of aminoglycoside, gentamicin (for 7 days) in combination with doxycycline (for 45 days) (DG) has been shown to have a lesser relapse rate than streptomycin (for 14 days) and doxycycline (for 45 days) (DS) (69). Thus, DG could be a better therapeutic option than DS combination for the treatment of human brucellosis.

Fluoroquinolones can be used in combination with rifampicin or doxycycline in case of non-complicated acute cases as an alternative combination to DR (45). Trimethoprim-sulphamethaxazole (TMP-SMX) was tested in endemic areas in combination with doxycycline or rifampicin; however, both regimens showed high treatment failures as well as relapse rates and thus are not considered as a good therapeutic option for the treatment of brucellosis in adult individuals (151). Because of the risk of irreversible staining of teeth, doxycycline cannot be used for the treatment of brucellosis in children (less than 9 yrs of age) instead a widely used regimen includes the combination of either TMP-SMX with gentamicin or rifampicin but this is still associated with high relapse rates (6, 94, 164).

In special cases, like pregnancy, rifampicin is the first choice of treatment while some studies suggest the use of TMP-SMX with or without rifampicin (92, 131). However, use of TMP-SMX in pregnant women has been associated with kernicterus (a rare neurological condition occurs in newborns with severe jaundice).

For the treatment of more complicated cases like neurobrucellosis and osteoarticular complications, duration of the therapy is the most important factor. Patients with spondylitis often require protracted antibiotic treatment, sometimes exceeding 12 months, with pharmaceutical and surgical treatment (171). Quinolones provide a better option for the treatment of spondylitis because of their ability to penetrate and achieve significant concentrations in bone and soft tissues (133). Use of ciprofloxacin in combination with doxycycline has been shown to be an effective treatment for spondylitis caused by *Brucella*. Brucellar endocarditis is a very complicated situation but fortunately very rare. The treatment requires surgery and post antibiotic treatment for 3-15 months usually utilizing at least three antibiotics effective against *Brucella* (144). In case of neurobrucellosis, currently there is no guideline regarding duration and therapeutic options. The duration may vary from 8 weeks to 2 years and often requires at least three antibiotics, principally doxycycline, rifampicin and TMP-SMX (16, 71). Use of streptomycin and tetracycline combination is not preferred because of their inability to reach high CNS concentrations.

In summary, there are conventional therapeutics for the treatment of brucellosis but still the duration of treatment is very long. Moreover, the percentage treatment failures and relapse cases still remains high. Therefore, there is a need for an alternative treatment that may be able to reduce the duration of treatment and thus related toxicity and patient non-compliance and finally treatment failures and relapses. If the duration of treatment could be reduced, then it might be possible to treat infected animals especially in the countries like India where slaughter of cattle is not possible due to

cultural norms and infected animals and their products remain a constant source of infection to humans.

Nanomedicine and *Brucella* treatment: Nanotechnology is the science of studying and manipulating materials at the nanometer scale, roughly between 1-500nm (174). At these size ranges, materials possess higher surface to volume ratio compared to the same material in micro- or macro-scales. These properties lead to the manipulation of these nanoscale materials in the fields of catalysis, electronics, robotics and medicine. It has long been desired to target therapeutic drugs to organs, cells and subcellular compartments, as well as to prolong their circulation time and reduce their toxic effects. But it is not possible to modify the drug itself to achieve these goals due to the loss of the drug's activity. Nanomedicine presents the possibility to load the drug molecule into nanoparticles and meet these goals. The surface of these carriers can be modified and attached to targeting molecules like proteins or antibodies. Slow release of the drug would increase the circulation time and slow down its elimination. Moreover, just by changing the size of nanoparticles, their fate inside the host can be altered. For example: small particles between 5-10 nm in diameter are readily eliminated from the body by the kidneys. However particles bigger that 10 nm are not eliminated by this means. Particles between 10-70 nm are suitable for subcutaneous injection as they can easily pass through small blood capillaries (70). Slightly larger particles between 70-200 nm show the best circulation time (179). Particles greater than 200 nm in diameter often end up in the spleens as a result of mechanical filtration and are eventually cleared by the phagocytic system (125).

Treatment of intracellular pathogens is often a challenge because of their intracellular localization. The drugs might fail to cross cell membrane barriers, get inactivated at low pHs found in endosomes or do not accumulate sufficiently to reach therapeutic concentrations. Nanomedicine presents an avenue to deliver drugs against intracellular pathogens like *Mycobacterium* spp. and *Brucella* spp. A drug like gentamicin is very effective in culture medium to kill *Brucella* but poorly crosses cell membrane barriers, gets inactivated by low endosomal pH and remains ineffective against intracellular *Brucella* (98). Previous studies have shown the potential of poly (lactide) and poly (lactide co-glycolide) (PLA and PLGA) nanoparticles loaded with gentamicin to treat *B. melitensis* infections *in vitro* and *in vivo*. In the initial studies, these nanostructures were found to cause embolism and death in mice because of agglomeration of particles (139). Later, different ratios of D and L isoforms of PLGA were used to synthesize gentamicin loaded microparticles and nanoparticles. Although microparticles were found to be effective with respect to the killing of *Brucella* in infected mice compared to free gentamicin, the encapsulation efficiency was very low.

In our laboratory, we have tested polymeric nanoparticles loaded with doxycycline and streptomycin against *B. melitensis* in mice (163). There was significant difference in the clearance of *Brucella* from infected mice treated with two doses of drug loaded nanoparticles compared to free drugs but no difference in efficacy was found after 4 or 6 doses (unpublished data).

Immunotherapy to treat brucellosis: Immunotherapy aims to target the host immune system rather than disease-causing agent (96). This strategy is of utmost importance in

case of cancer treatment and chronic bacterial disease where drugs fail to act and a stronger host immune response is required (82). During chronic *Brucella* infections there is a reduction in host immune responses against *Brucella*. Therefore, enhancement of the host immune response by immunostimulants along with antibiotics could help in clearing *Brucella* from the host (4). It has been shown that T lymphocytes from chronic brucellosis patients show reduced blastogenesis in presence of phytohaemagglutinin (a T cell mitogen), *E. coli* LPS or heat killed *Brucella (167)*. Conventional therapy to treat *Brucella* infection leads to an increase in T-cell blasts at the end three months therapy (138). Thus, T-cell anergy in chronic *Brucella* cases leads to the switching from Th1 to Th2 immune response facilitating chronic infection. An immunostimulant that can reverse this anergy may help in clearing *Brucella* from the host.

Immunostimulating drugs for the treatment of chronic intracellular bacterial infections are broadly of three types: bacterial preparations, synthetic immunostimulating agents and natural immunostimulants like lymphokines (reviewed in (182)). Two types of immune response stimulators have been reported previously for the treatment of brucellosis, levamisole and IFN-γ. Levamisole is an antiparasitic agent with cellular immunostimulatory effects. Studies conducted with chronically *Brucella* infected human patients showed that levamisole added to antibiotics resulted in serological and clinical improvement (83, 145). However, a study conducted on mice failed to show any clearance of *Brucella* from the infected organs after treatment with levamisole (194). Also a recent report (35) showed co-administration of levamisole along with conventional antibiotics therapeutics had no effect to improve anergy against *Brucella* in

chronically *Brucella* infected human patients. No immune stimulating effect of levamisole was found on the basis of phagocytic ability and lymphocyte subgroup ratios in the patients.

As described previously, IFN-γ is a key component required to provide protection against *Brucella*. Administration of IFN-γ has been shown to improve therapy against *Mycobacterium* infections, including cases of multi-drug resistance strains (32, 175, 183). In some reports, use of IFN-γ showed better improvement in clinical and immunological parameters in *Brucella* infected patients than levamisole but its use is restricted due to nonspecific host stimulation and rapid elimination of IFN-γ from the host (138). In a comparative study, human brucellosis cases were treated either with doxycycline-rifampicin alone or in combination with IFN-α or levamisole. IFN-α was administered subcutaneously three times a week for 6 months and levamisole was given as 450 mgs tablets orally every day, two days per week for 6 months. It was found that patients treated with both the chemotherapeutic and the immunostimulator showed higher levels of leukocyte migration inhibition factor (MIF). However, levels were higher in the IFN-α treated cases than in the levamisole treated group. Improved clinical symptoms and decreased *Brucella* antibody were observed in IFN-α treated cases than levamisole treated group. Pathogen-specific immunostimulants, like DNA vaccines, have been shown to be effective along with therapeutics in cases of mice infected with *M. tuberculosis* and to induce specific immune responses against the pathogen (68). No such report has been published in case of *Brucella* treatment yet. The current vaccines cannot be used to enhance the immune response in already infected hosts, as most are live attenuated and restricted to animals due to safety concerns. Thus, a subunit

vaccine or DNA vaccine or a strain of *Brucella* that is known not to cause disease would be worth testing to determine if an immunostimulator can be used to treat chronic brucellosis.

Rationale of dissertation research

Brucellosis is the most common zoonotic disease worldwide (33). There is no recommended regimen to treat animals, as it is not economical. Human brucellosis treatment requires 6-8 weeks of combination therapy but still has up to 1-5% treatment failure and a 5-10% relapse rate (169).

This dissertation research has two focuses with the overall aim of developing a better therapeutic regimen for the treatment of chronic *Brucella* infection in a mouse model. Doxycycline is the first choice of drug for the treatment of human brucellosis. However, monotherapy results in higher relapses thus combination therapy is recommended. Rifampicin-doxycycline (DR) combination is widely used but has been shown to result in higher relapses than doxycycline-aminoglycoside combinations (69). As a choice of aminoglycoside, treatment with gentamicin in combination with doxycycline (DG) has shown fewer relapses than doxycycline-streptomycin (DS) combination (170). Drugs like gentamicin are very effective against *Brucella* in culture medium but poorly crosses cell membrane barriers and remain ineffective against intracellular *Brucella* (98). Gentamicin cannot cross the hydrophobic core of biological membranes because of its positive charges (140). It is taken up by the cells by endocytosis and mostly ends up in endosomal-lysosomal compartments where their efficacies if further reduced due to low pH (110).

The first aim of the research presented herein was to develop a delivery system to deliver gentamicin intracellularly and to reach sufficient intracellular concentrations of the drug in a bioactive state. In order to achieve these goals, drug-delivery system (DDS) based approaches were explored. Drug-loaded nanoparticles have been shown before to overcome cell membrane barriers and deliver drug intracellularly (136). Gentamicin-loaded nanoparticles were synthesized and tested both *in vitro* and *in vivo* against *B. melitensis.*

The second aim was to activate host immune system against chronic *Brucella*. *Brucella* modulates the host immune system and hides inside host cells leading to chronic infections. In human infections there is a switch from Th1 to Th2 that occurs in chronic cases (141) but there is no evidence of a similar switch in mice. In mice it has been shown that there is no class switching but infected spleens lose their architecture and most CD4+ and CD8+ cells and thus cannot eliminate *Brucella* infected cells (76). In either scenario boosting the host immune response may help in clearing infection. Stimulation of the immune system through non-specific immunostimulants like cytokines can lead to long-lasting systemic toxicity, non-specific immune responses and activation of immunosuppressive immune mechanisms (182). Most *Brucella* vaccines approved for animal use can provide specific immunostimulation but are live attenuated and are not considered safe for use in humans. The present research focuses on using specific immunostimulants in the form of either a subunit vaccine (OMVs) or irradiated *Brucella* species (*B. neotomae)*; both products are not known to cause disease.

I tested the comparative efficacy of free drug vs. drug encapsulated in nanoparticles to treat a *B. melitensis* infection both *in vitro* and *in vivo*. I investigated the efficacy of

gentamicin containing polymeric block ionomer complexes (BICs) and gentamicin loaded magnetite block ionomer complexes (MBICs) as a drug delivery vehicle for the elimination of *Brucella*. Toxicity caused by these nanoparticles was also accessed.

In addition I also tested the efficacy of Pluronic P85 as an adjuvant to enhance the potential of outer membrane vesicles (OMVs) obtained from *Brucella* as a vaccine against *B. melitensis* challenge in mice.

Lastly, I explored the combined effect of antibiotics and vaccines on treatment of *B. melitensis* infection in mice. As a vaccine I included irradiated *B. neotomae* and OMVs+P85 while a doxycycline + gentamicin combination was used as the antibiotics.

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Chapter 2.1

Block ionomer complexes as a tool to deliver gentamicin intracellularly against *Brucella melitensis*

Abstract

More than 500,000 new cases of human brucellosis occur annually worldwide. Drugs like aminoglycosides remain ineffective against intracellular *Brucella* due to their limitation to cross cell membrane barriers. In the present study we tested the efficacy of block ionomer complexes (BICs) of polyether-polyacrylate block copolymers and gentamicin as a tool for intracellular drug delivery. Gentamicin loaded BICs showed intensity average hydrodynamic diameters of 170-340 nm. Up to 42 wt% gentamicin was complexed in the BICs. Release kinetics of gentamicin from BICs showed an initial burst release of drug followed by a steady release up to 40-60 hr. No significant toxic effect or macrophage activation was determined when J774A.1 murine macrophage-like cells were incubated with BICs at a concentration range from 0.25 mg/ml to 0.625 mg/ml. Compared to free drug (0.5 Log_{10}), significant reduction in intracellular *B. melitensis* was achieved in infected macrophages treated with BICs (2.78 Log_{10}). Administration of BICs did not cause any toxicity in the mice. Compared to free gentamicin, mice treated with BICs alone or in combination with doxycycline did not show any significant difference in clearance of *Brucella* from spleens and livers.

Introduction

Brucellosis caused by *Brucella spp*. is the most common zoonotic disease worldwide. The pathogen is easily spread from animals to humans in aerosols and is thus classified as a Biosafety Level-3 (BSL3) pathogen (7). Although vaccines are available for animal use, so far there is no effective vaccine for humans (10). After entering the host, *Brucella* preferentially targets the monocytic-macrophagic system (5). *Brucella* can not only survive inside phagocytic cells but also replicate, causing chronic infection in the host (3). Prolonged treatment is required to treat human brucellosis that often results in patient non-compliance and toxicity (1). Rates of treatment failure and relapse associated with brucellosis are 1-5% and 5-10% respectively. A combination of tetracycline with rifampicin or aminoglycosides is recommended for the treatment of *Brucella* infection in humans. It has been shown that a doxycycline-gentamicin combination results in fewer relapses compared to doxycycline-streptomycin combination (23). Gentamicin is highly effective against *Brucella* in culture medium but remains ineffective against intracellular *Brucella*. Three factors that affect the efficacy of gentamicin against intracellular *Brucella* are its poor ability to cross cell membrane, inactivation at low endosomal pH and insufficient intracellular accumulation (9, 20, 22). Either liposome or polymeric drug loaded nanoparticles have been shown to be readily taken up by phagocytic cells and reach organs with phagocytic cells (4, 11). This presents an avenue to target intracellular pathogens like *Brucella* that reside inside phagocytic cells. (6). In the present study we investigated the potential of block ionomer complexes (BICs), made up of anionic polyether-polyacrylate block copolymers complexed with cationic aminoglycoside as a vector for intracellular drug delivery.

Efficacy of these block ionomer complexes (BICs) was tested *in vitro* and *in vivo* against *B. melitensis* infection.

Materials and Methods

Bacterial strains and cell lines: *B. melitensis* 16M was routinely grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 37 ^{0}C and 5% CO_2. For determining the cell cytotoxicity, cell stimulation and *in vitro* efficacy of BICs, J774A.1 murine macrophage-like cells (American Type Culture Collection, ATCC) were used. These cells are routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomicin in a humidified 5% CO_2 atmosphere at 37°C.

Synthesis and characterization of BICs: Diblock and pentablock co-polymers of poly(ethylene oxide) (PEO), poly(propylene oxide) (PPO) and poly(acrylic acid) (PAA) were synthesized by controlled free radical polymerization (ATRP) process as described before (Manuscript PAT-11-150.R2 accepted, Polymers for Advanced Technologies). Gentamicin sulfate (0.134 g, 0.082 g gentamicin, 1.69 x 10^{-4} mol gentamicin, ~8.45 x 10^{-4} eq of cations) was dissolved in 25 mL of PBS and the pH was adjusted to 5.0 with 1M NaOH to make a solution concentration of 3.28 mg/mL of gentamicin. $CH_3O\text{-}PEO_{45}\text{-}PAA_{117}$ (0.050 g, 5.53 x 10^{-4} eq of anions) and $PAA_{27}\text{-}PEO_{27}\text{-}PPO_{41}\text{-}PEO_{27}\text{-}PAA_{27}$ (0.050 g, 3.03 x 10^{-4} eq of anions) were dissolved separately in 25 mL of PBS and the pH of the solution was adjusted to pH 5.0 with 1M NaOH, yielding a 4 mg/mL solution of polymer. Fabrication of the complexes was conducted in a custom-built, small continuous mixer outfitted with four inlets with syringe pumps for introducing solutions at

specified rates (Figure 2.1.1). Two inlets were injected with 15 mL of PBS at a flow rate of 5.00 ml/min, while the other two inlets were injected with the drug solution and polymer solution, each at a flow rate of 12.0 mL/min. The Reynold's number was 7,900, which corresponded to a mixing time of <50 ms. Final concentrations in the stream exiting the mixer were 1.41 mg/mL of polymer and 1.16 mg/mL gentamicin. The resultant dispersion of BICs was collected and immediately centrifuged with a Thermo-Scientific Sorvall centrifuge at 3750 rpm (relative centrifugal force ~ 3000 x g) for ~45 min or until all of the elute had passed through a Millipore centrifugal filter equipped with a 3000 g/mol MWCO cellulose acetate membrane. The solid complexes were collected on the membrane filter. The complexes were dispersed in a minimal volume of DI water (~10 mL) to remove them from the membrane filter and then freeze-dried.

The size of nanoplexes was determined using dynamic light scattering (DLS) at 37°C in PBS at a concentration of 1.0 mg/mL immediately after fabrication. Intensity average diameters are reported as an average of three measurements. The concentration of gentamicin in the complexes was determined using a colorimetric *o*-phthalaldehyde assay as described previously (2).

<u>Macrophage culture - cell cytotoxicity and nitric oxide quantification:</u> To determine any possibility of cell cytotoxicity caused by the polymer or gentamicin loaded BICs, Cell titer 96® Aqueous Non-Radiocative Cell proliferation MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay (Promega Inc.) was used. Briefly, approximately 2 x 10^5 J774A.1 cells/well, resuspened in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic, were seeded in 96 well

tissue culture plate. After 24 hr of incubation, media was removed and free drug, free copolymer (PAA_{27}-PEO_{27}-PPO_{41}-PEO_{27}-PAA_{27} and CH_3O-PEO_{45}-PAA_{117}) or BICs were added to cells at different concentrations along with the DMEM media containing 10% FBS. The cells were incubated for 24 hr. Next day, cell culture supernatants were removed and collected in another 96 well plate to perform Griess assay (described later). Cells were washed with PBS to remove traces of drug or polymer. After washing, 100 µL of fresh media was added to the cells. To each well 20 µL of Cell Titer 96® Aqueous reagent solution was added except for negative control wells. The plate was incubated in dark for 2-3 hr at 37°C in a humidified 5% CO2 atmosphere. The absorbance at 490 nm was recorded using a 96-well ELISA plate reader. Percentage cell viability was calculated considering the OD values of the cells incubated only with media as 100% cell viability.

A Greiss assay was used to determine the presence of nitric oxide in the cell culture supernatants. In this assay, nitrite, an end product of nitric oxide oxidation, is measured in cell culture supernatants. Greiss assay reagents included 1% (wt/vol) sulfanilamide (Sigma-Aldrich) and 0.1% (wt/vol) naphthylenediamine dihydrochloride (Sigma-Aldrich). Both reagents were dissolved separately in 2.5% phosphoric acid. Immediately after collecting the cell culture supernatant (described above), 50 µL of both Griess assay reagents were added to an equal volume of the culture supernatant. Serial dilutions of sodium nitrite beginning at 250 µM to 0.5 µM were used as a standard. After adding the reagents to the wells absorbance was measured at 550nm using an ELISA plate reader. The levels of nitric oxide were calculated using the formula obtained from the standards using SoftMax Pro software (Molecular Devices). Cells incubated with *E. coli* LPS (500

ng/mL) and media alone were the positive and negative controls respectively. Nitric oxide was also measured in the supernatants from the cells incubated with LPS along with BICs or free polymer.

In vitro Infection Assay: Efficacy of the BICs compared to free drug to kill intracellular *Brucella* was tested in J774A.1 macrophages. Macrophages were seeded at a density of 5 x 10^6 cells/well in a 24-well cell culture plate (Corning Inc.) 24-36 hr prior to infection. At 90% confluency in the wells, the cells were infected with *B. melitensis* for 1 h at a multiplicity of infection of 1:100. After phagocytosis, the media was removed and fresh media containing 50 µg/mL gentamicin was added and the cells were incubated for 45 min to kill extracellular bacteria. The media was removed and the infected cells were incubated with DMEM + 10% FBS for 24 hr to set up the infection model. At 24 hr post-infection, the cells were washed twice with DMEM and 50 µL of either free drug or the BIC complexes, suspended in DNAase RNAase free water to achieve a drug concentration of 1mg/ml was added to the infected cells along with 2 mL of DMEM + 10% FBS and they were incubated further for 24 hr. The media was removed and the cells were washed twice with PBS. To determine the intracellular *Brucella* load, the cells were lysed using 250 µL of 0.1% Triton X-100™ and 10-fold serial dilutions of lysates were prepared and spread on TSA plates. Colony forming units (CFUs) of *Brucella* were determined after incubating the plates for 48 hr at 37°C under 5% CO_2.

In vivo infection and treatment: Almost 6-8 weeks old female BALB/c mice (Harlan Laboratories, USA) were used as a model for *B. melitensis* infection. Mice were infected

with 2 x 10^4 CFUs/mouse of *B. melitensis* injected intraperitonially (i.p). A total of 35 mice (5 mice per group) were treated after 6 weeks of infection with gentamicin alone (either as free drug or nanoplexes, 5 mg/kg body wt. i.p., 6 doses, once per day) or in combination with doxycycline (3 mg/kg body wt. i.p., 10 doses, once per day). Mice treated with saline were the negative controls. The mice were euthanized 48 hr after last treatment. The livers and spleens were collected and *Brucella* CFUs were determined by plating serial dilutions of the organ homogenates on TSA plates.

Results

Characterization of BICs: Gentamicin loaded BICs showed a mean intensity diameter of 170-340 nm. The drug encapsulation efficiency was between 40-42 wt%. Release study showed a burst release of gentamicin from the complexes soon after dissolution and then a steady release up to 40-60 hr (Manuscript PAT-11-150.R2 accepted, Polymers for Advanced Technologies).

Macrophage culture - cytotoxicity and nitric oxide production: The BICs toxicity was measured using J774A.1 macrophages and MTT assay. Figure 2.1.2 shows that BICs had no adverse effect on the cell viability, in the range of concentrations studied. Cells treated with BICs showed almost the same percentage viability as shown by the control cells. Furthermore, the cells maintained normal morphology (data not shown). The effect of BICs on nitric oxide production by macrophages was analyzed using a standard Griess assay (Figure 2.1.3 shows representative data). Upon incubation for 24 hr with BICs, macrophages did not show any production of nitric oxide. While as expected, addition of LPS to the culture medium induced nitric oxide production. BICs

did not affect the levels of nitric oxide production when added to the cells along with LPS.

In vitro efficacy of the BICs against Intracellular *Brucella*: Efficacy of the BICs compared to free drug against intracellular *Brucella* was studied in J774A.1 macrophages. Significant bacterial reductions of 2.78 Log_{10} were obtained when infected cells were treated with BICs (Figure 2.1.4). This reduction was significantly higher than the reduction by free drug (0.75 Log_{10}). Infected cells treated with polymer alone did not show any reduction of intracellular *Brucella*.

In vivo efficacy of the BICs to clear *B. melitensis* infection in mice: Efficacy of BICs was tested in BALB/c mice infected with *B. melitensis.* Mice were treated either with BICs alone or in combination with doxycycline. Doxycycline is the first drug of choice to treat *Brucella* infection in humans (1). Combination therapy with either doxycycline-rifampicin or doxycycline-aminoglycoside has been shown to be more efficacious than monotherapy. When used alone (Table 2.1.1), no reductions in *Brucella* CFUs were achieved either in spleens or livers of mice treated with BICs compared to non-treated controls or those treated with free gentamicin. Mice treated with BICs in combination with doxycycline showed better clearance of *Brucella* compared to control mice (Table 2.1.1) but not better than mice treated with doxycycline alone or with free doxycycline and free gentamicin combination. No signs of nephrotoxicity were observed on the histopathological examination of kidneys from the mice treated with BICs (data not shown).

Discussion

Our objective was to develop gentamicin-loaded nanoparticles that can deliver antibiotic into *Brucella* infected cells. Several groups have investigated biodegradable polyester carriers for aminoglycosides without substantial success (13, 14, 18). Gentamicin loaded poly(D,L-lactide) and poly(lactide-*co*-glycolide) (PLGA) nanospheres and microspheres delivered higher concentrations of gentamicin in the livers and spleens of *Brucella* infected mice compared to free drug but only low concentrations of ~1 wt% of the drug could be incorporated into carriers (14). This is attributed to the inherent incompatibility of the polar drug with the relatively hydrophobic polyesters. In contrast, high concentrations of gentamicin (up to 46 wt%) have been loaded into microspheres comprised of polyanionic hyaluronate complexed with cationic chitosan (15, 16). The high aminoglycoside loading capacities of the hyaluronates is encouraging for our development of electrostatically-bound carriers. Several researchers have also incorporated gentamicin into liposomes but liposome instabilities are a concern since break-up tends to release encapsulated drugs prematurely (8, 17).

In the present study, we blended a hydrophilic diblock polymer PEO-*b*-PAA^{-+}Na with ampiphilic pentablock polymer PAA^{-+}Na-*b*-PEO-*b*-PPO-*b*-PEO-*b*-PAA^{-+}Na and cationic gentamicin to prepare BICs. The strategy was to obtain nanostructures with an ampiphilic outer shell. Hydrophobicity provided by poly(propylene oxide) (PPO) should aid in keeping the particles dispersed. While hydrophilicity provided by poly(ethylene oxide) (PEO) should increase biocompatibility (21) and prevent inter-particle agglomeration. The anionic core of the nanostructures made by poly(acrylic acid) (PAA) encapsulated the cationic gentamicin through ionic interaction and provided a pH dependent release of drug inside cells. To form complexes with desirable compositions

utilizing a blend of the diblock and pentablock copolymers we used a multi-inlet vortex mixer (MIVM) to enable rapid, turbulent mixing of the polymer and drug solutions (Figure 2.1.1). This results in nucleation of the BICs from a homogeneous supersaturated solution and also provides a means for maintaining the ratio of polymer to drug constant throughout the process. Very high drug encapsulation efficiency (up to 42%) was achieved using this technique.

BICs did not cause any cell cytotoxicity when incubated with macrophages. It should be noted that the concentrations of BICs that were tested *in vitro* were much higher than the concentrations used to treat *Brucella* infected macrophages. Nitric oxide was measured in macrophage cell culture supernatants as an inflammation marker. As expected, LPS induced production of nitric oxide by macrophages. But macrophages incubated with BICs did not produce any detectable nitric oxide. Moreover, there was no change in the nitric oxide production by macrophages stimulated with LPS upon co-incubation with BICs. This shows that BICs do not promote any inflammatory action when in contact with macrophages. Our results show that gentamicin delivered via BICs cleared *Brucella* significantly more efficiently than free drug from infected macrophages. This clearance proves that BICs were able to deliver bioactive drug intracellularly. This shows that the complexes are an effective transporter for these polar antibiotics into the cells. Contrary to *in vitro*, BICs failed to clear *Brucella* from infected mice. Whether tested alone or in combination with doxycycline, administration of BICs to the *Brucella* infected mice did not result in better clearance of *Brucella* compared to free gentamicin. We speculate that inefficacy of BICs in mice could be due to the initial burst release of the drug from these complexes. Most of the drug was released from the complexes in

less than 10 hours of dissolution. Thus, after administration in mice the drug was released from BICs before it could reach the targeted organs like spleens and livers. If the entire drug was released from BICs soon after administration then it should act like free gentamicin. Gentamicin in combination with doxycycline provided significantly better clearance of *Brucella* both in spleens and livers compared to doxycycline alone. But mice treated with BICs in combination with doxycycline showed no difference in clearance of *Brucella* compared to mice treated with doxycycline alone. It might be possible that most of the injected BICs were taken up by the circulating monocytes and did not reach the infected cells in spleens and livers. Study of biodistribuition of these nanoplexes in the mice could be helpful in understanding the fate of nanoplexes inside mice. Future work should focus on synthesizing more stable nanoparticles that could hold the drug more tightly and will have a steady release of drug over time. Moreover, drug loaded nanoparticles should be fluorescently tagged so that the biodistribuition and fate in mice can be studied using *in vivo* imaging techniques (12, 19).

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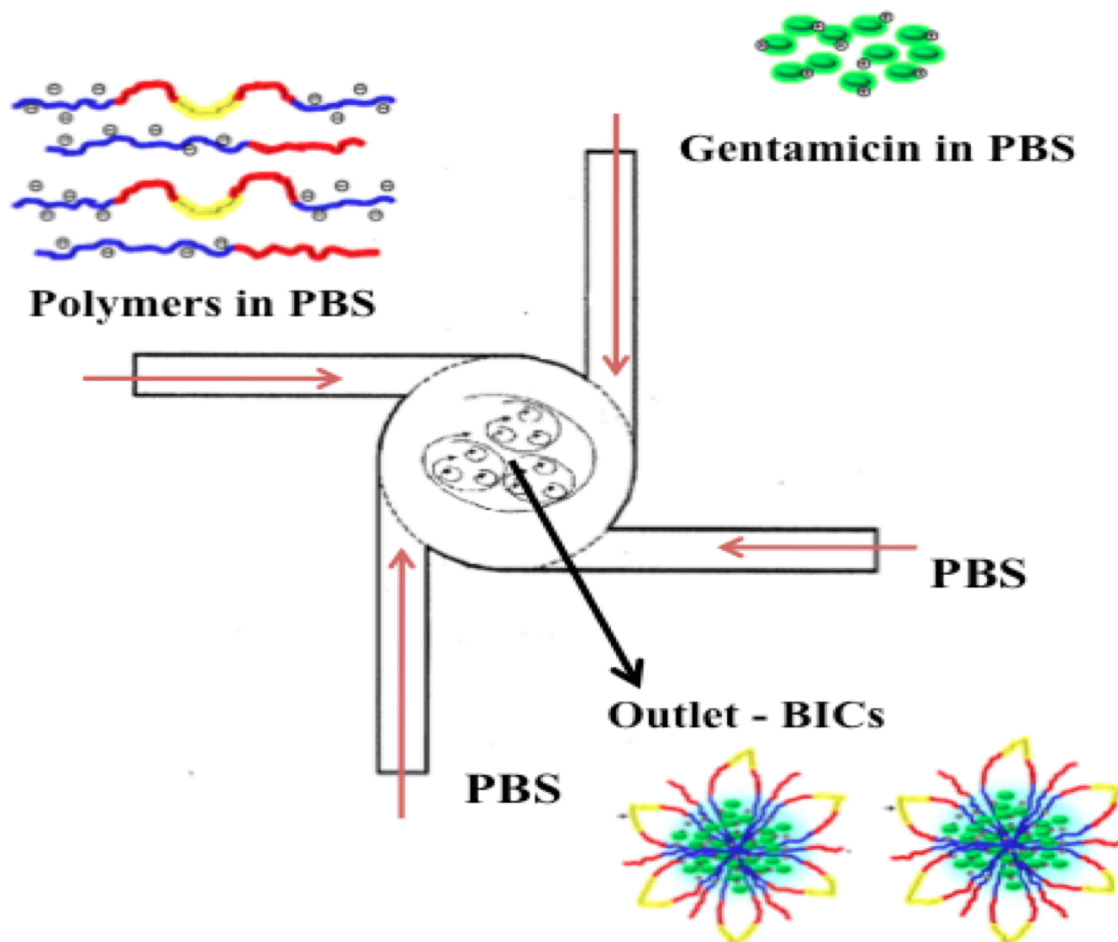

Figure 2.1.1: Four-inlet vortex mixer for the continuous fabrication of BICs core shell.

BICs were formed utilizing blends of pentablock and diblock copolymers and gentamicin dissolved in PBS

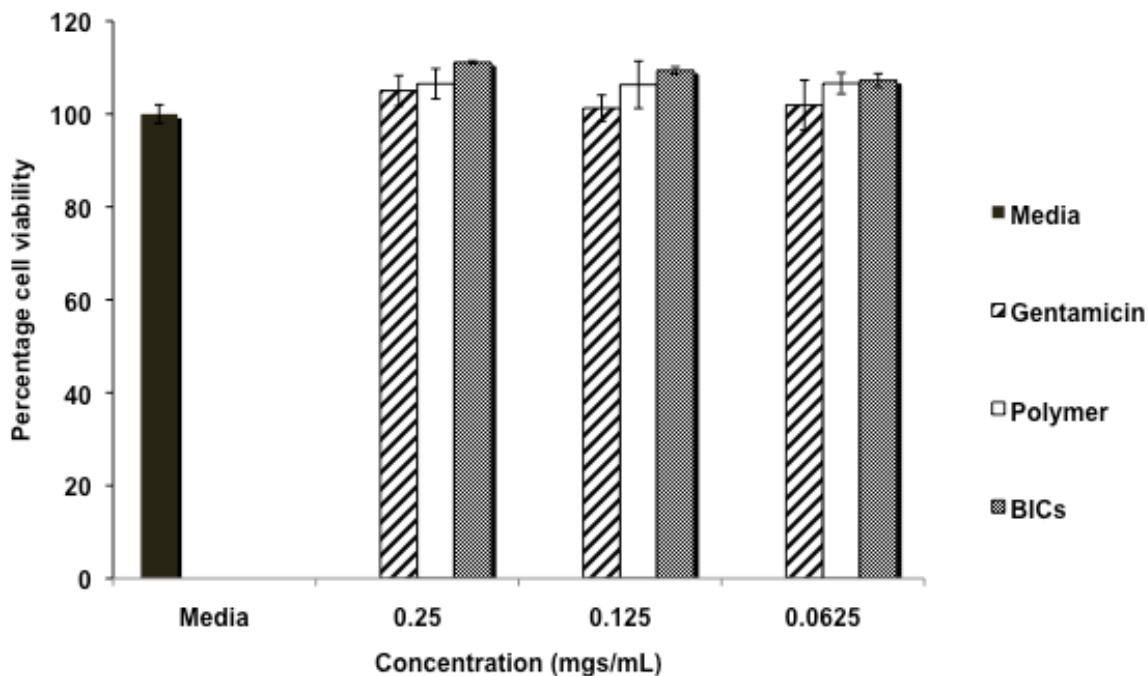

Figure 2.1.2: ***In vitro*** **toxicity of BICs.**

J774A.1 macrophages were incubated with different concentration of gentamicin, either free or encapsulated in BICs and with empty BICs dissolved in media for 24 hr. All the samples were tested in triplicates. Cells incubated with media only were the positive controls. After 24 hr of incubation viability of cells was measured using MTS assay. The cell viability from the optical density values was calculated considering the cell viability of control groups as 100%. The data represent the mean ± SEM of two independent experiments.

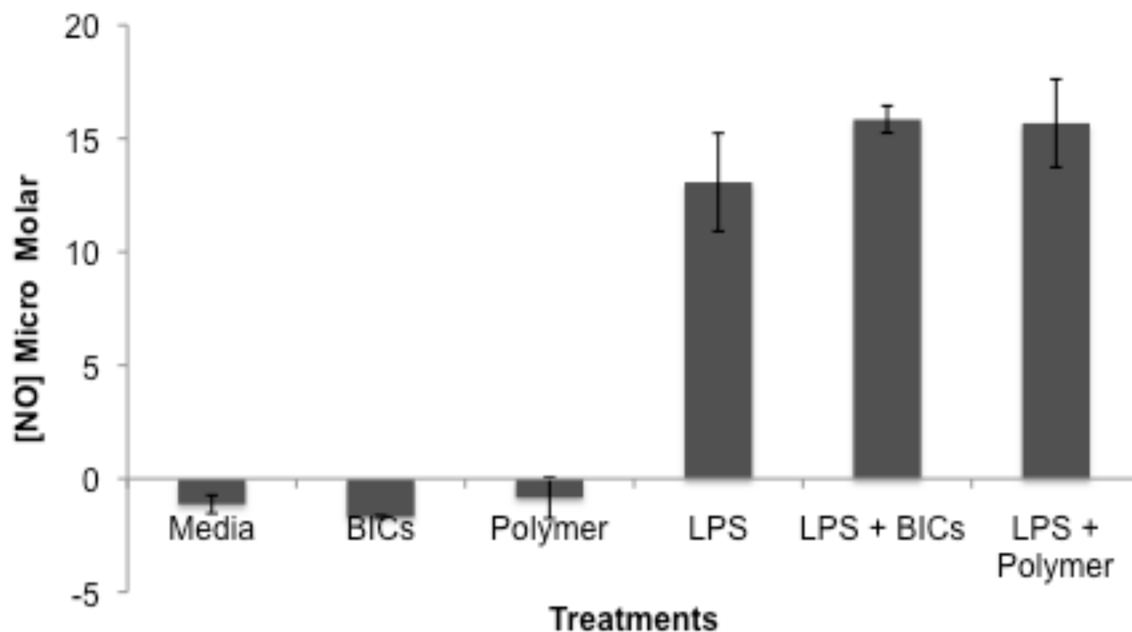

Figure 2.1.3: Production of nitric oxide by macrophages incubated with BICs.

J774A.1 cells were incubated with BICs fabricated at pH 5.0 and pH 7.4 and empty BICs (polymer) either in the presence or absence of LPS. Cells incubated with media alone and with LPS alone were the positive controls for the non-stimulated and stimulated groups respectively. After 24 hr of incubation, levels of nitric oxide were measured in culture supernatants using a standard Griess assay. Data represent mean ± SEM of three independent samples.

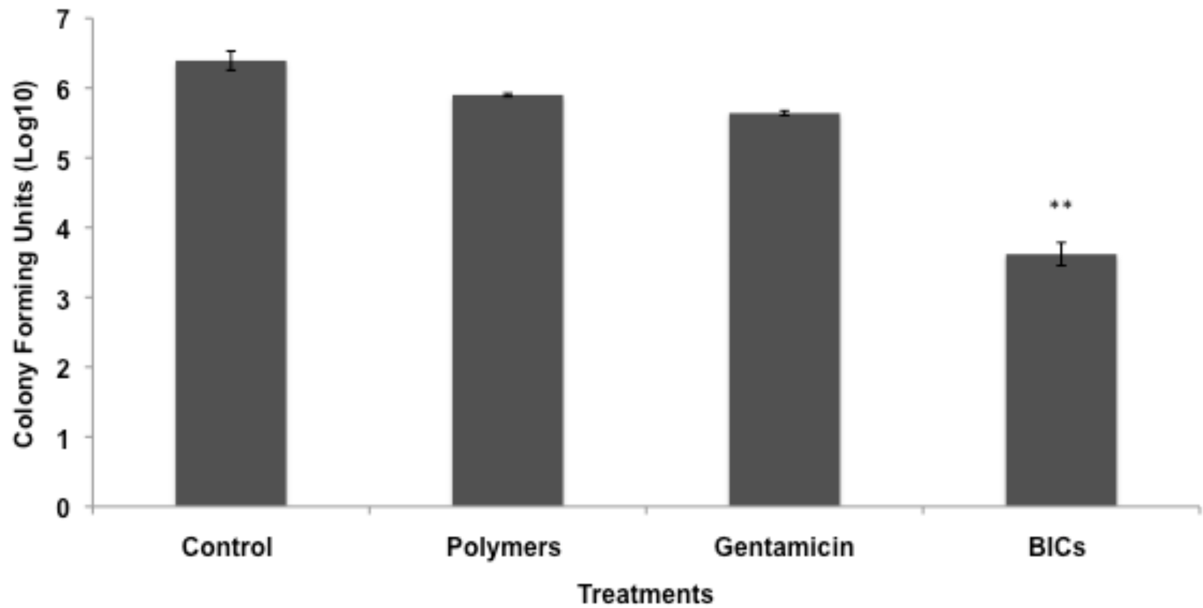

Figure 2.1.4: *In vitro* efficacy of BICs against *B. melitensis*.

J774A.1 cells were infected with *B. melitensis* (MOI 1:100) and treated with gentamicin either free or encapsulated in BICs. Free polymer was also tested. Non-treated cells were the negative control. All the treatments were tested in triplicates. The data represents the mean ± SEM of $\log_{10}$ *Brucella* CFUs and double asterisk (**) indicates the statistically significant difference from control and gentamicin treated groups.

Table 2.1. 1: *In vivo* efficacy of BICs against *B. melitensis*

Treatment (6 doses)	Mean Log_{10} CFUs/spleen ± SD	Mean Log_{10} CFUs/liver ± SD
Saline	5.68 ± 0.08	4.12 ± 0.08
Gentamicin	5.70 ± 0.2	4.18 ± 0.34
BICs	5.49 ± 0.21	4.11 ± 0.17

Treatment (10 doses)	Mean Log_{10} CFUs/spleen ± SD	Mean Log_{10} CFUs/liver ± SD
Saline	5.76 ± 0.21	3.65 ± 0.24
Doxycycline	4.32 ± 0.06*	2.51 ± 0.43*
Doxycycline + Gentamicin	3.8 ± 0.27**	1.86 ± 0.44**
Doxycycline + BICs	4.27 ± 0.08*	2.23 ± 0.43*

Mice were infected with *B. melitensis* and allowed to develop chronic infection. Top panel shows the efficacy of BICs to clear *Brucella* from infected mice compared to free gentamicin. Bottom panel shows the efficacy of BICs in combination with doxycycline to treat *Brucella* infection in mice. Saline treated mice were the negative controls. The data represent the mean ± std dev of Log_{10} *Brucella* CFUs in spleens and livers of mice in each group (n=5). Single asterisk (*) and double asterisks (**) represents statistically significant difference ($p \leq 0.01$) from the saline treated mice and doxycycline treated mice respectively.

Chapter 2.2

In vitro and *in vivo* efficacies of gentamicin loaded magnetite block ionomer complexes against *Brucella melitensis* infection

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Abstract

Anionic copolymers can enable intracellular delivery of cationic drugs such as gentamicin which otherwise cannot efficiently cross cell membrane barriers. The efficacy of magnetite block ionomer complexes containing gentamicin (MBICs) was tested both *in vitro* and *in vivo* against *B. melitensis*. Anionic block copolymers were used to coat nanomagnetite through adsorption of a portion of anions on the particle surfaces, then the remaining anions were complexed with 30-32 weight percent of gentamicin. The zeta potential changed from -39 mV to -13 mV after encapsulation of the drug with complimentary charge. The MBICs had intensity average hydrodynamic diameters of 62 nm while the polymer-coated nanomagnetites without drug were 34 nm in size. No toxicity as measured by MTS assay was observed upon incubation of the MBICs with J774A.1 murine macrophage-like cells. Confocal microscopic images show that the MBICs were

taken up by the macrophages and distributed in the cell cytoplasm and endosomal/lysosomal compartments. Upon treatment with MBICs, *B. melitensis* infected macrophages showed significantly higher clearance of *Brucella* compared to the treatment with free gentamicin. However, these *in vitro* efficacies were not translatable to the *Brucella* infected mice after treatment with MBICs. Histopathological examination of kidneys from the MBICs treated mice revealed multifocal infiltration of macrophages containing intracytoplasmic iron (MBICs) admixed with lymphocytes in peri-renal adipose.

Introduction

Brucellosis, caused by *Brucella spp*., is the most common zoonotic disease worldwide.[1] There are six classical species of which three can cause disease in humans, namely *B. melitensis, B. suis* and *B. abortus*. Brucellosis is difficult to diagnose because of non-specific symptoms and signs that prevent timely management and treatment of the disease.[2] *Brucella* infects both phagocytic and non-phagocytic cells where they survive and multiply intracellularly, making treatment difficult.[3] Although vaccines are available for animals, there is no effective vaccine for humans.[4] The efficacy of the animal vaccine is limited by many factors including improper and limited use. Antibiotic treatment of brucellosis is not economical for animals. The antibiotic regimen for humans is a combination therapy for ≥6 weeks recommended by the World Health Organization (WHO).[5] Tetracyclines are typically used in combination with aminoglycosides, rifampicin, trimethoprim-sulfamethoxazole or quinolones to

treat human brucellosis. However, treatment failure and relapse rates are as high as 1-5% and 5-10% respectively.[6]

Aminoglycosides like gentamicin are very effective *against Brucella* grown in culture medium, but due to their positive charges they permeate hydrophobic cell membranes very slowly [7] and thus are ineffective against intracellular *Brucella.*[8] Aminoglycosides at high doses can result in nephrotoxicity and/or ototoxicity and thus should be used within safe limits.[9,10] Drug delivery systems (DDS) capable of crossing cell membrane barriers have been reported before but were limited either by instability or agglomeration of the complexes.[11,12] We have previously reported the efficacy of gentamicin loaded block ionomer complexes (BICs) to kill *Brucella in vitro* (Manuscript PAT-11-150.R2 accepted, Polymers for Advanced Technologies). However, BICs loaded with gentamicin failed to treat *B. melitensis* infection in mice (unpublished data). We speculated that the burst release of the encapsulated antibiotic in the BICs hampered the efficacy in mice. Moreover, these nanoplexes could not be imaged in mice to reveal *in vivo* distribution. In the present effort, we compared the efficacy of gentamicin loaded magnetite block ionomer complexes (MBICs) and free gentamicin against intracellular *Brucella*. Magnetite nanoparticles are very powerful contrast enhancement agents for T2 weighted magnetic resonance imaging (MRI).[13,14] Also, because of their dark color MBICs can be visualized in tissue sections. We hypothesized that the presence of magnetite in MBICs would enhance our ability to track them *in vivo* and that, by the virtue of their size and surface properties, MBICs would be taken up by phagocytic cells and deliver drug intracellularly. MBICs showed a

slow release of gentamicin over a period of 40-60 hr (Pothayee N., 2011, manuscript submitted) and no burst release of drug was observed. This paper describes the efficacy of MBICs to deliver gentamicin to kill intracellular *B. melitensis* as well as and the toxicity of MBICs both *in vitro* and *in vivo*.

Materials and methods

Synthesis and characterization of MBICs: All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Poly(ethylene oxide) and poly(acrylic acid) are designated as PEO and PAA respectively.

a) Synthesis of a PEO-*b*-PAA and PEO-*b*-PAA-NH_2 block ionomers: mPEO-Br or *tboc*-NH-PEO-Br was used as a macroinitiator for polymerization of *t*-butyl acrylate through controlled free radical polymerization (ATRP) [15] for the synthesis of PEO-*b*-PAA and amine functionalized PEO-*b*-PAA (PEO-*b*-PAA-NH_2) respectively. Briefly, mPEO-Br or *tboc*-NH-PEO-Br (M_n 2,150 g mol^{-1}, 1.075 g, 5.0 x 10^{-4} mol), *t*-butyl acrylate (9.75 mL, 6.0 x 10^{-2} mol), and 9 mL dry toluene were added into a 50-mL Schlenk flask. After degassing, 72 mg of copper bromide (Cu(I)Br, 5.0 x 10^{-4} mol) and 0.100 mL of pentamethyldiethylenetriamine (PMDETA, 5.0 x 10^{-4} mol) were added quickly under nitrogen. The reaction mixture was deoxygenated with 3 freeze-thaw cycles, and then heated at 80°C for 12 hr. After the polymerization, the catalyst was removed by filtering the reaction mixture through neutral alumina using dichloromethane as the eluent. The solvent was evaporated and the block copolymer was dried under vacuum at 50°C overnight. Removal of the t-butyl ester groups and the *tboc* group was achieved by dissolving the *tboc*-NH-PEO-*b*-PtBA (2.0 g, 1.09 x 10^{-2} eq of t-butyl

ester groups) in 50 mL of dichloromethane. Trifluoroacetic acid (5 ml, 6.50×10^{-2} mol) was added and the reaction was stirred at room temperature for 24 hr. The polymer was precipitated in hexane and dried under vacuum, then dissolved in THF and dialyzed against 4 L of DI water through a cellulose acetate membrane (MWCO 3,500 g mol^{-1}) for 24 hr. The PEO-*b*-PAA or H_2N-PEO-*b*-PAA and with PEO M_n of ~2,000 g mol^{-1} and PAA M_n of ~7,200 g mol^{-1} was recovered by freeze-drying.

b) Synthesis of a PEO-*b*-PAA-NH_2 labeled with fluorescein isothiocyanate (FITC): PEO-*b*-PAA-NH_2 (2K-7.2K) (0.4 g, 0.05 mmol), FITC (9.7 mg, 0.025 mmol) and DMF (2 mL) were added to an aluminum foil covered 20-mL vial equipped with a stir bar in dark. The reaction was conducted at room temperature overnight under dark conditions.

c) Synthesis of nanomagnetite: $Fe(acac)_3$ (2.14 g, 8.4×10^{-3} mol) and benzyl alcohol (45 mL, 0.43 mol) were charged to a 250-mL, three-neck, round-bottom flask equipped with a water condenser and overhead stirrer, then placed in a Belmont metal bath with thermostatic (± 1°C) control. The solution was dehydrated at 110°C for 1 h under N_2. The temperature was raised to 205°C and maintained for 40 hr. The reaction was cooled to RT and the particles were collected by centrifugation (4000 rpm, 30 min). The magnetite nanoparticles were washed 3X with acetone (100 ml each), then were dispersed in chloroform (20 ml) containing oleic acid (0.30 g). The solvent was removed under vacuum at RT, and the oleic acid-coated nanoparticles were washed 3X with acetone (50 mL each) to remove excess oleic acid. The particles were dried under vacuum at RT,

then stored as a dispersion in chloroform at a concentration of 20 mg/mL prior to use.

d) Synthesis of magnetic block ionomer complexes (MBICs and MBICs-FITC): Homogeneous ligand adsorption from an organic solvent was employed to assemble block ionomers onto nanomagnetites. A representative method for preparing a targeted composition of 66:34 % by weight polymer to magnetite is provided. Oleic acid-stabilized magnetite nanoparticles (50.0 mg) were dispersed in chloroform (10 mL) and charged to a 50-mL round-bottom flask. To synthesize MBICs, mPEO-*b*-PAA (100.0 mg) was dissolved in DMF (10 mL) and added to the dispersion. To synthesize MBICs-FITC, 50 mg of mPEO-b-PAA and 50mg of mPEO-b-PAA-FITC were used. The mixture was sonicated in a VWR 75T sonicator for 4 hr under N_2, and then stirred at RT for 24 hr. The nanoparticles were precipitated in hexanes (200 mL). A permanent magnet was utilized to collect the magnetite nanoparticles and free oleic acid was decanted with the supernatant. The particles were dried under vacuum overnight, then dispersed in DI water (20 mL) with adjustment of the pH to ~ 7 with 1 N NaOH and sonicated for 30 min. The particles were dialyzed against DI water (1 L) for 24 hr in a 25,000 g mol^{-1} MWCO dialysis bag to remove free polymer or free FITC. The dispersion was filtered through a 0.2 μm Teflon filter to sterilize it and to remove any aggregates. A black-brown solid product was obtained after freeze-drying.

e) Preparation of gentamicin loaded MBICs or MBICs-FITC: MBICs or MBICs-FITC (40 mg, 2.18×10^{-4} eq COOH) and gentamicin sulfate solution in 10 mM phosphate buffer pH 7.2 (45 mg gentamicin sulfate, 27 mg gentamicin, 5 mL)

were mixed in a glass vial. The solution was sonicated for 5 min and transferred to a centrifugal filter unit equipped with a cellulose acetate membrane (MWCO of 10,000 $gmol^{-1}$). The free drug and salt solution were removed by centrifuging the dispersion at 3750 rpm for 1 h. This allowed the liquid to pass through the membrane into the bottom of the centrifugation unit, and the particles were collected on the membrane. They were removed from the membrane by re-dispersing them in a small amount of DI water (10 mL), and then the dispersion was freeze-dried to obtain the final product.

The amount of gentamicin loaded into the MBICs was determined using the *o*-phthalaldehyde assay as previously described.[15] The size and zeta potential of MBICs were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS.

Bacterial strain and cell lines: For *in vitro* and *in vivo* infection studies, *B. melitensis* 16M (stock cultures, Virginia Tech) was used. *B. melitensis* 16M was grown in tryptic soy broth (TSB) and tryptic soy agar (TSA) at 37°C and 5% CO_2. For cell culture studies, J774A.1 murine macrophage-like cells (ATCC) were grown in Dulbecco's Modified Eagle's media (DMEM, Sigma) with 10% heat inactivated fetal bovine serum and 1% Penicillin-Streptomycin (Pen-strep, Cellgro) at 37°C in a humidified 5% CO_2 atmosphere.

In vitro cytotoxicity: Cell titer 96® Aqueous Non-Radioactive Cell Proliferation MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

2H-tetrazolium) Assay Solution (Promega) was used to access the cytotoxicity of the empty MBICs and gentamicin loaded MBICs. Briefly, ~2 x 10^5 J774A.1 murine macrophage like cells/well, suspended in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin, were seeded in 96-well tissue culture plates (Corning Inc.). After 24 hr of incubation, the medium was removed and either free drug, empty MBICs or gentamicin loaded MBICs were added to cells at different concentrations along with the DMEM medium containing 10% FBS and incubated for 24 hr. Cells incubated only with medium were the positive controls for the assay. All the samples were tested in triplicate. The cells were washed with PBS to remove traces of drug or polymer and 100 µL/well of fresh medium was added to the cells. Cell Titer 96® Aqueous reagent solution (20 µL) was added to each well except to the negative controls. The plate was incubated in the dark for 2-3 hr at 37°C in a humidified 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using a 96-well ELISA plate reader. The cell viability was calculated considering the absorbance value of the control group as 100% cell viability.

Confocal micrscopy: For confocal microscopic examination, 1 x 10^5 J774A.1 cells/well were seeded in 10 mm diameter microwells of 35 mm petri dishes (Mat-tek Corporation). The cells were incubated with 2 mL DMEM + 10% FBS for 24 hr. Next day, the medium was changed and fluorescently labeled MBICs (25 µg/well) resuspended in culture medium, were added to the cells and incubated for an hour at 37°C and 5% CO_2. Cells incubated with medium alone were the

negative controls. To study colocalization, an image-iT live lysosomal and nuclear labeling kit (Invitrogen) was used to stain the lysosome/endosome and nuclear compartments. The wells were examined under a 40x water-immersion objective on a Ziess LSM 520 META confocal microscope.

In vitro infection assay: The efficacies of the MBICs compared to free drug to kill intracellular *Brucella* were tested in J774A.1 cells. Macrophages were seeded at a density of 5×10^6 cells/well in a 24-well cell culture plate (Corning Inc.) 24-36 hr prior to infection. At 90% confluency in the wells, the cells were infected with *B. melitensis* for 1 h at a multiplicity of infection of 1:100. After phagocytosis, the medium was removed and fresh medium containing 50 µg/mL gentamicin was added and the cells were incubated for 45 min to kill extracellular bacteria. The medium was removed and the infected cells were incubated with DMEM + 10% FBS for 24 hr to set up the infection model. At 24 hr post-infection, the cells were washed twice with DMEM and 50 µL of either free drug or the MBICs, suspended in DNAase RNAase free water to achieve a drug concentration of 1 mg/mL, was added to the infected cells along with 1.95 ml of DMEM+10% FBS (final drug concentration 25 µg/mL, either free or in MBICs) and were incubated further for 24 hr. The medium was removed and the cells were washed twice with PBS. To determine the intracellular bacterial load, the cells were lysed using 250 µL of 0.1% Triton X-100™ and 10-fold serial dilutions of lysates were prepared and spread on TSA plates. Colony forming units (CFUs) were determined after incubating the plates for 48 hr at 37°C under 5% CO_2.

In vivo infection and treatment: Six to eight weeks old female BALB/c mice (Harlan Laboratories, USA) were used as a model for *B. melitensis* infection. Mice were infected with 2 x 10^4 CFUs of *B. melitensis* injected intraperitonially (i.p.). After 6 weeks of infection, mice were divided into 4 groups (5 mice per group) and were treated with doxycycline alone (3 mg/kg body wt. i.p.) or in combination with gentamicin (either as free drug or encased in MBICs, 5 mg/kg body wt. i.p.) for 10 days (once daily). The mice were euthanized 48 hr after the last treatment. The livers and spleens were collected and *Brucella* CFUs were determined by plating the serial dilutions of the organ homogenates on TSA plates.

Histopathology: To assess the *in vivo* toxicity of free gentamicin and MBICs, kidneys from untreated and treated (doxycycline-gentamicin and doxycycline-MBICs) mice were harvested. Kidneys were then fixed in 10% neutral buffered formalin, routinely processed, embedded into paraffin blocks, and 5 μm sections were stained with hematoxylin and eosin on glass slides (Virginia-Maryland Regional College of Veterinary Medicine, Veterinary Teaching Hospital). Slides were blindly examined by two pathologists using light microscopy and were scored on a scale of 0-4 on the basis of the lesions present (inflammation, necrosis, degeneration, hemorrhage or edema) as reported before.[16,17] Scores were given separately on the basis of lesions in the kidney and peri-renal adipose tissue. Lesions included inflammation (infiltration of inflammatory cells),

necrosis (morphologic changes of renal parenchymal cells consistent with degeneration and necrosis) and vascular disruptions (hemorrhage, edema and other signs of vascular leakage). Scores were defined as follows: 0 = unremarkable, 1= minimum, 2= mild, 3= moderate and 4 = marked. Kidneys from the *B. melitensis* infected mice were collected after 10 doses or 28 doses of the doxycycline in combination with gentamicin (either free or MBICs) and examined to assess the effect of shorter and prolonged therapy using MBICs. Cohen's Kappa Coefficient (*k*) was determined to calculate the agreement between two pathologists based on the standard values. According to the standard, k = 0.75 is excellent, between 0.40 and 0.75 is fair to good and below 0.40 represents poor agreement.

Results

Synthesis and characterization of MBICs: Gentamicin loaded MBICs were successfully synthesized according to figure 2.2.1. Dynamic light scattering showed that the intensity average diameter of MBICs was 62 ± 4 nm. The charge characteristics of empty MBICs (without gentamicin) had a zeta potential of -38.6 ± 10.1 mV and it decreased to -13.4±7.4 mV after encapsulation of the cationic gentamicin. The amount of gentamicin encapsulated was found to be ~ 32% w/w, which is very high compared to the drug loading efficiencies reported previously using alternative strategies.[18-20]

In vitro cytotoxicity: Compared to the untreated controls, no significant toxicity was determined by different concentrations of MBICs (either empty or gentamicin loaded) upon incubation with J774A.1 macrophages (Figure 2.2.2). It should be noted that the concentrations used were much higher than the required therapeutic concentrations. No morphological changes were observed in cells after incubation with MBICs (data not shown).

Intracellular trafficking: Confocal microscopy revealed an efficient uptake of the MBICs by the macrophages (Figure 2.2.3B). Figure 2.2.3C suggests that MBICs resided in endosomes/lysosomes and also in the cytoplasm within macrophages. As macrophages have some auto-fluorescence, it was important to visualize the cells incubated with media alone (negative controls) under the same fluorescent intensity used to visualize MBICs inside the cells. Figure 2.2.3A shows the absence of any fluorescence in the cells exposed to same fluorescent intensity used to obtain image 2.2.3B. This confirms that the fluorescence in 2.2.3B is that of the fluorescently labeled MBICs.

In vitro efficacy against *B. melitensis*: Compared to free gentamicin, MBICs cleared significantly higher number of *B. melitensis* from infected macrophages (Figure 2.2.4). Empty MBICs showed no effect on clearance of *Brucella* from infected macrophages. These results suggest that MBICs were not only able to deliver gentamicin intracellularly, but also that the drug was able to reach the intracellular niche where *Brucella* was replicating.

In vivo efficacy and toxicity: The efficacy of gentamicin loaded MBICs compared to free gentamicin against *B. melitensis* was tested in chronically *Brucella*-infected mice. Neither free gentamicin nor MBICs showed a reduction in *Brucella* counts in the spleens and liver compared to untreated controls in infected mice (data not shown). When used in combination with doxycycline, both free gentamicin as well as MBICs significantly enhanced the clearance of *Brucella* from the spleens and livers of infected mice compared to doxycycline alone (Table 2.2.1). To the authors' knowledge this is the first time the efficacy of doxycycline alone and the doxycycline + gentamicin combination has been compared in a mouse model chronically infected with *B. melitensis*. While both combinations (doxycycline + gentamicin and doxycycline +MBICs) showed better efficacy than doxycycline alone, there was no significant difference between the efficacies of the two combinations.

Histopathological examination of kidneys from the treated mice shows no renal lesions after 10 doses of free doxycycline either alone or combination with free gentamicin or MBICs (data not shown). Furthermore mice treated with 28 doses of drug, showed minimal lesions based on the scores given after pathological examinations (Table 2.2.2 and 2.2.3). There was good to substantial agreement (k= 0.656, n=48) between the scores given by two pathologists. Lesions in kidney and peri-renal adipose were scored separately. The most common lesions seen in mice treated with MBICs were the deposition of brown pigment laden-macrophage infiltrates admixed with lymphocytes near the periphery of the peri-

renal adipose. Special iron stains (Perl's Prussian blue) revealed the cytoplasmic pigment contained iron and small extracellular iron deposits between adipocytes. The cellular responses in these cases are consistent with a foreign body response or cell mediated immunity to an exogenous substance.

Discussion

The capability of delivering drugs directly to the targeted organs, cells and subcellular compartments has been long desired to increase drug efficacy and to reduce its toxicity. However, it is not possible to modify the drug itself to achieve these goals due to the possibility of losing drug activity. Nanomedicine presents the potential to load the drug molecule into nanoparticles as a drug-delivering vector to meet these goals.[21] The surfaces of these vectors can be modified and attached to targeting molecules like proteins or antibodies.[22] Encapsulation of drug in nanoparticles exhibits slow release of the drug that in turn increases the drug circulation time and slows its elimination. It has been shown before that liposomes and polymeric nanoparticles are readily taken up by the circulating monocytes for their clearance and that they localize in organs with high phagocytic activity (reviewed in [11]). This makes them useful to target drugs against intracellular pathogens especially those residing inside phagocytic cells. We have previously reported the efficacy of block ionomer complexes to deliver gentamicin intracellularly (Manuscript PAT-11-150.R2 accepted, Polymers for Advanced Technologies). The high gentamicin loading efficiencies were achieved by strong interactions between the cationic gentamicin and anionic polymer. The same drug-binding strategy was applied for the synthesis of MBICs

and, consequently, 32% w/w drug-loading efficiency was achieved. Moreover, the poly(ethylene oxide) (PEO) portion of the block ionomers comprised the outer shell of the MBICs. PEO is hydrophilic in nature and was chosen as a segment of the block copolymer to keep particles dispersed by providing steric repulsion. It is well documented that the presence of PEO on the nanoparticle surfaces decreases opsonization of nanoparticles and prolongs their circulation time.[23-25]

In the present study we have shown the uptake of gentamicin loaded MBICs by macrophages. No cellular toxicity was detected when J774A.1 macrophages were incubated with MBICs (Figure 2.2.2). The concentrations tested were far higher than the concentrations used to treat *Brucella* infected macrophages *in vitro*. Measuring toxicity of these complexes is of major importance in order to verify that the intracellular pathogen is killed by the delivered drug and not because of the toxic effect of the vector.

Confocal microscopic images revealed the efficient uptake of the MBICs by macrophages (Figure 2.2.3B). Further, we have shown the co-localization of MBICs in phagosomal/lysosomal compartments (Figure 2.2.3C). Some of the MBICs were also found within the cell cytoplasm, which is beneficial for drug delivery to subcellular compartments such as *Brucella* containing vacuoles. We still do not know whether or not co-localization in the phagosomes/lysosomes affects the bioactivity of encapsulated gentamicin due to the acidic environment. However, *in vitro* clearance of *Brucella* from the infected macrophages treated with MBICs shows that an adequate amount of active gentamicin was delivered inside the infected cells. Free gentamicin showed very little effect on intracellular

Brucella counts compared to non-treated cells. This might have resulted from the killing of extracellular *Brucella* by gentamicin and prevented cell-to-cell transmission of *Brucella* during the 24-hr incubation following the treatment. Empty MBICs did not result in the clearance of intracellular *Brucella* in the *in vitro* model. This demonstrates that the effect shown by MBICs against *Brucella* was only attributable to delivered gentamicin and that the vector was inert.

When gentamicin (either free or loaded in MBICs) was tested to treat chronic *Brucella* infection in mice, there were no reductions in *Brucella* counts in spleens and livers compared to non-treated controls (data not shown). Later, gentamicin was used in combination with doxycycline to treat chronic *Brucella* infection in mice. Doxycycline in combination with gentamicin, either free or loaded in MBICs, showed significantly higher clearance both in spleens and livers compared to doxycycline alone. Based on symptomatic and laboratory examinations, the doxycycline-gentamicin combination has been shown to be a better therapeutic for human brucellosis than doxycycline monotherapy.[26,27] Here, we clearly show that the doxycycline-gentamicin combination resulted in better clearance of *Brucella* from infected mice. However, there was no difference in *Brucella* clearance in mice treated with free gentamicin vs. MBICs in combination with doxycycline.

It has been reported previously that particles between 70-200 nm show the longest circulation time after intravenous administration in host.[28] Particles greater than 200 nm in diameter often end up in the spleens as a result of mechanical filtration and are eventually cleared by the phagocytic system.[29] The

lack of efficacy of MBICs against *Brucella* in mice might be because of shorter circulation time of MBICs owing to their small size (64 nm). Future work should focus on increasing the size of the MBICs and testing for their *in vivo* efficacy to kill *Brucella.* It has been shown before that nanoparticles with amphiphilic outer shells are taken up more readily than hydrophilic-shelled particles.[30] MBICs in the present study had hydrophilic outer shells due to the presence of PEO. We speculate that changing the outer shell to have amphiphilic properties may increase the uptake of particles by phagocytic cells.

As mentioned before, gentamicin causes nephrotoxicity. It has been shown that gentamicin delivered through polymeric nanoparticles causes less toxicity than free gentamicin due to slower release of the drug.[31] The histopathology assessed the nephrotoxicity caused by gentamicin released from the MBICs compared to the free gentamicin. With respect to pathological scores, there was no difference between MBICs and free gentamicin. The ability to track the nanoparticles in a host helps to address many concerns about the fate of nanoparticles. Histopathology of the kidneys obtained from mice treated with 28 doses of doxycycline and MBICs showed pigment-laden macrophages (Figure 2.2.5a) within the periphery of peri-renal adipose tissue. This macrophage cytoplasmic pigment was darker than hemosiderin yet lighter than melanin, which suggests an exogenous pigment. Further staining with Perl's Prussian blue revealed the presence of iron (presumably magnetite in MBICs) in the cytoplasm of the macrophages and extracellularly between adipocytes (Figure 2.2.5b). We speculate that after peritoneal injection, MBICs deposited along the visceral fat

(peri-renal adipose) resulted in macrophage and lymphocyte recruitment and phagocytosis. These macrophage and lymphocyte infiltrates are consistent with minimal to mild inflammation.

In conclusion, we were able to show that MBICs containing electrostatically-bound gentamicin are a potential drug delivery system for targeting intracellular *Brucella.* The black-brown color of MBICs made it possible to visualize them during histopathological examination of kidneys from euthanized mice. Magnetite nanoparticles in MBICs are powerful contrast enhancement agents for T2-weighted magnetic resonance imaging (MRI) because of their high magnetization, low toxicities and particulate nature.[32,33] Thus, magnetite resonance imaging (MRI) techniques should be used to study the *in vivo* distribution of MBICs in live mice. This will help to determine the fate of MBICs and to determine the reasons for its inefficacy against *Brucella* in mice.

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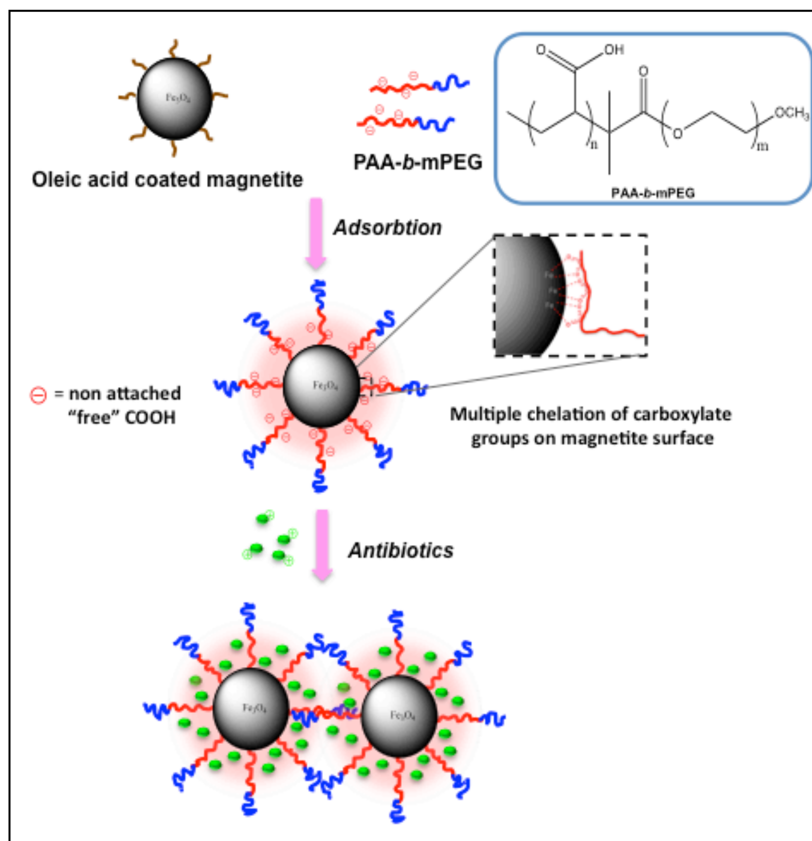


Figure 2.2.1: Schematic showing the step-wise synthesis of gentamicin loaded magnetite block ionomer complexes (MBICs).

The magnetite core was first synthesized via reductive decomposition of an iron (III) organo-metallic precursor, which allows formation of discrete and uniform particles. Well-defined nonionic-ionic block copolymers of monomethoxy-functional poly(ethylene oxide) (mPEO) and poly(acrylic acid) (mPEO-*b*-PAA) were synthesized through controlled free-radical polymerization (ATRP). The polymer was bound to the surfaces of the magnetic nanoparticles through ligand adsorption of the PAA component, thereby creating a double corona structure comprised of an outer nonionic PEO shell and an inner ionic layer of PAA. The portions of carboxylate groups that are not attached to the magnetite provide functionalities and binding sites for gentamicin via ionic complexation and chelation.

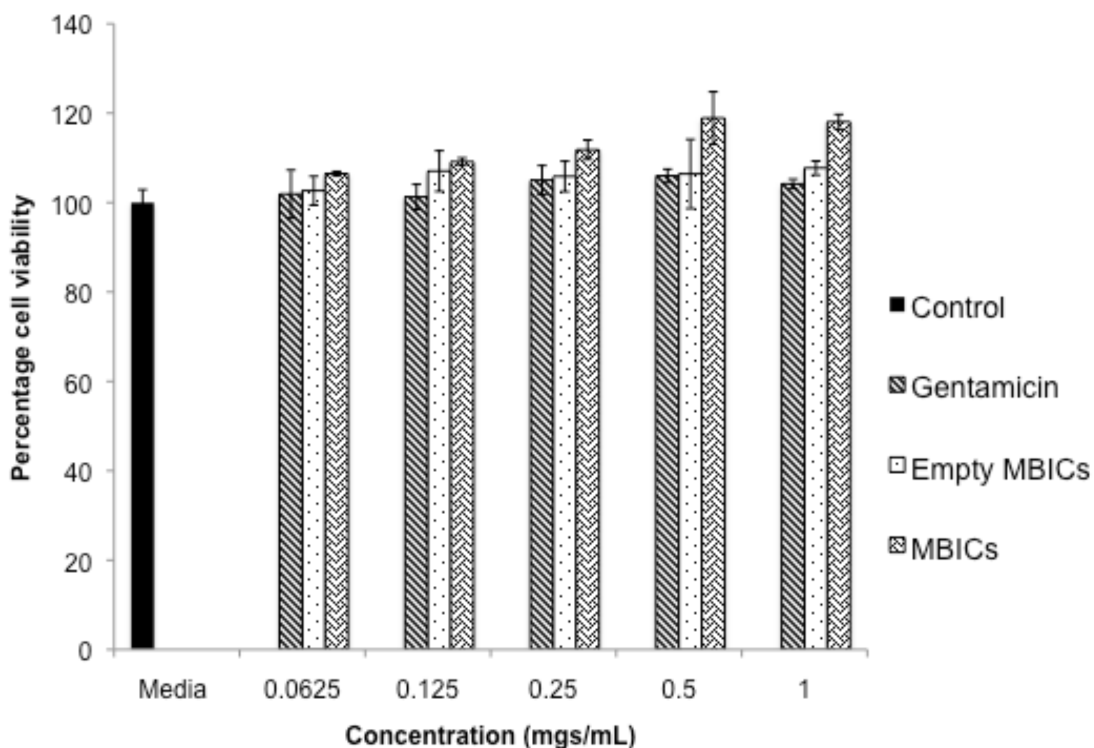


Figure 2.2.2: ***In vitro*** **toxicity of MBICs.**

J774A.1 cells were incubated with range of concentrations of free gentamicin, empty MBICs and MBICs. Cells incubated with media were the positive controls. All the treatments were tested in triplicate. After 24 hours of incubation, cell viability was determined using a MTS assay. Absorbance was measured at 450 nm and cell viability was determined considering the absorbance showed by positive controls as 100% cell viability. Data presented here represents mean ± SEM of three independent experiments.

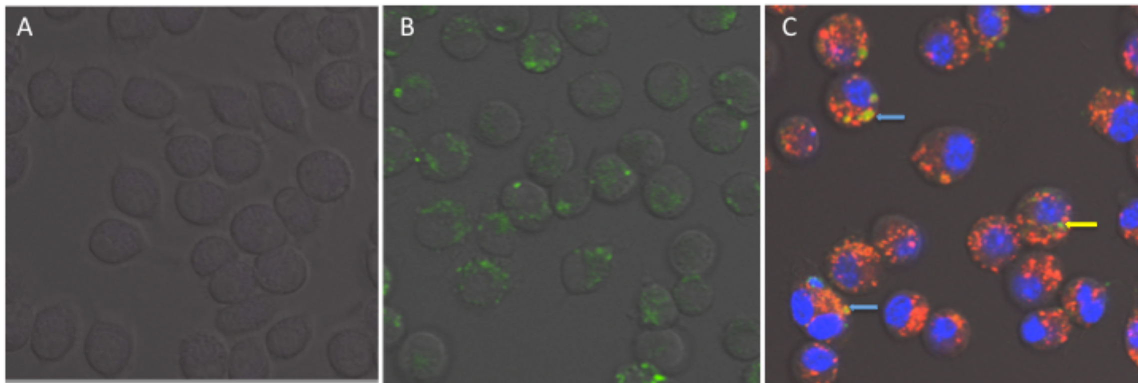


Figure 2.2.3: Confocal images showing the uptake and intracellular localization of MBICs.

J774A.1 cells were incubated with fluorescently labeled MBICs (green) and stained with nuclear stain (blue) and lysosome stain (red). Non-stained cells (image A) were the negative controls. Confocal image shows uptake of MBICs by J774A.1 macrophages (image B). Staining with nuclear stain and lysosomal stain shows the intracellular localization of MBICs (image C), blue arrows show fusion between lysosomes and MBICs, yellow arrow indicate the MBICs in cytoplasm of the cell.

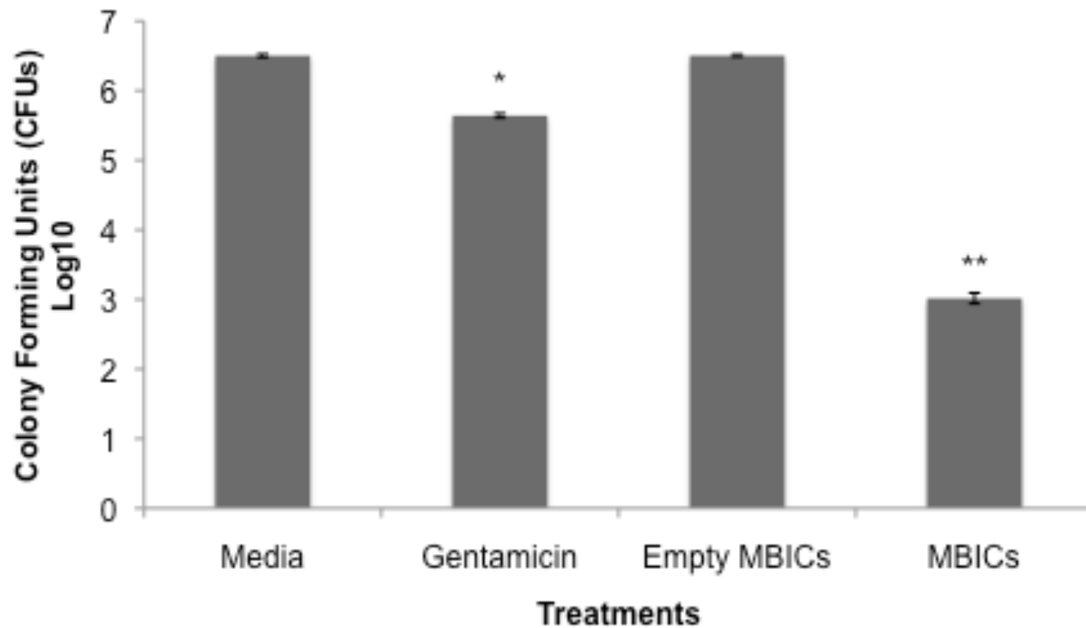
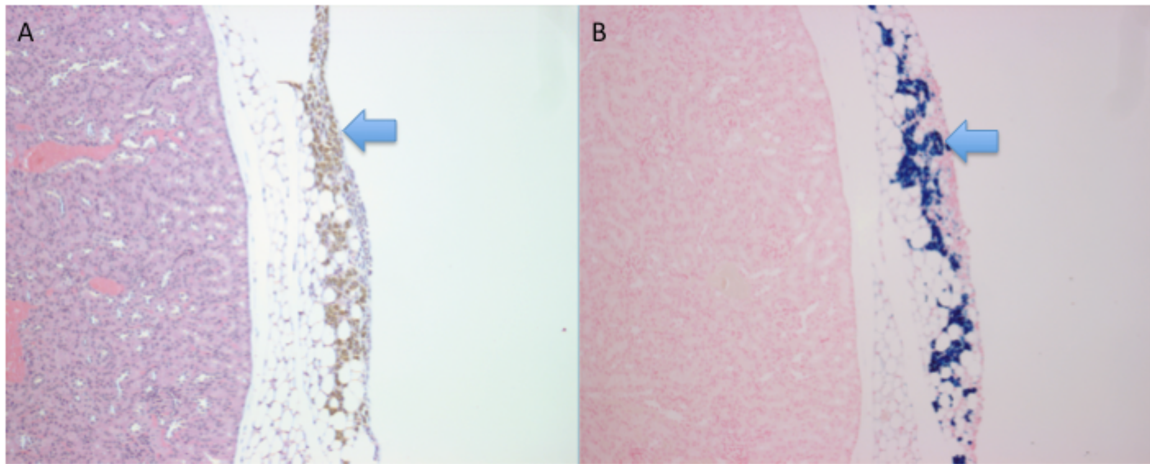


Figure 2.2.4: *In vitro* efficacy of MBICs compared to free gentamicin against *B. melitensis* inside J774A.1 cells.

J774A.1 cells were infected with *B. melitensis* and treated with gentamicin, either free or loaded in MBICs. Cells were lysed after 24 hr of treatment and intracellular *Brucella* CFUs were determined. Media treated cells were the negative controls. All the treatments were tested in triplicate. Data presented here represents mean ± SEM of two independent experiments. Single asterisk (*) represents statistically significant difference ($p \leq 0.001$) from negative controls and double asterisk (**) represents statistically significant difference ($p \leq 0.001$) from negative controls as well as from free gentamicin treated group.



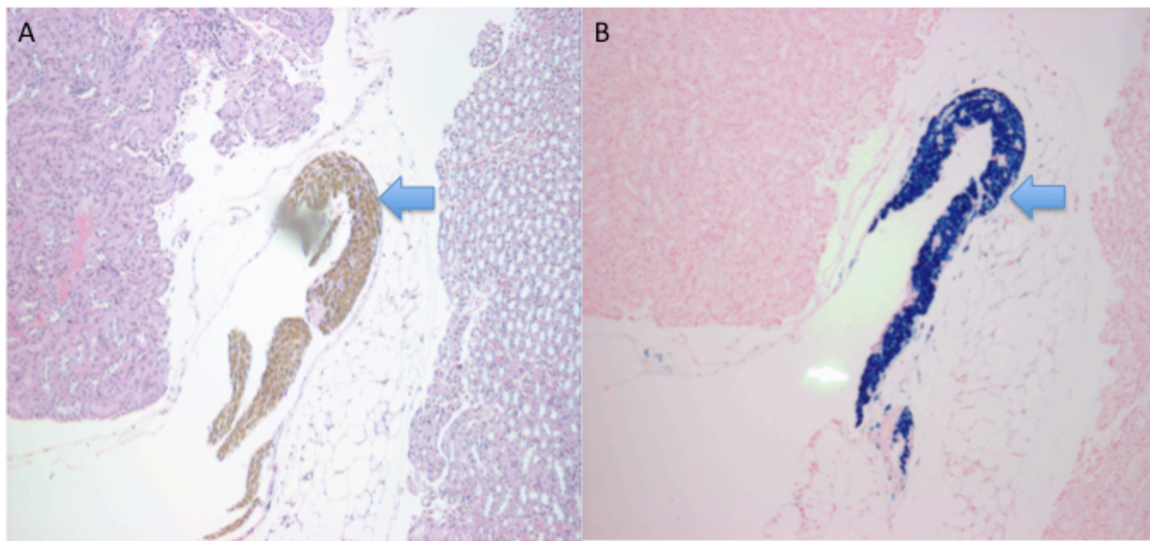


Figure 2.2.5: Kidney micrographs obtained from mice treated with doxycycline and MBICs.

Sections were stained with hematoxylin and eosin (5a), pigmented macrophages (arrow) in peri-renal adipose tissue. Sections stained with Perl's Prussian blue (5b) show the presence of intracytoplasmic iron (presumably MBICs) in the macrophages and extracellular between adipocytes

Table 2.2. 1: *In vivo* efficacy of MBICs in combination with free doxycycline against *Brucella melitensis.*

Treatments	CFUs (Log_{10}) in Spleens	CFUs (Log_{10}) in Livers
Saline	5.8 ± 0.21	3.7 ± 0.25
Doxycycline	4.3 ± 0.06*	2.5 ± 0.43*
Doxycycline + Gentamicin	3.8 ± 0.27**	1.9 ± 0.43**
Doxycycline + MBICs	4.1 ± 0.12**	1.8 ± 0.4**

Mice were treated with 10 doses (once daily) of doxycycline in combination with gentamicin (free or MBICs). Saline treated mice were the negative controls. Mice were euthanized and *Brucella* CFUs were determined in spleens and livers. Data presented here represents mean ± SEM (n=5). Single asterisk (*) represents statistically significant difference ($p \leq 0.01$) from saline treated mice and double asterisk (**) represents statistically significant difference ($p \leq 0.01$) from saline as well as from doxycycline treated mice.

Table 2.2. 2: Renal histopathological scores on examination of hematoxylin and eosin (H and E) stained sections of kidneys from MBICs treated mice.

Kidney number	Saline (Kidney score)		Doxycycline-Gentamicin (Kidney score)		Doxycycline-MBICs (Kidney score)	
	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2
1	0	0	0	1	0	0
2	0	0	0	0	0	0
3	0	0	1	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	1	1	0	0	0	0
8	0	0	0	0	0	0

Kidneys were examined by two pathologists and scored on the basis of lesions present. Lesions included inflammation (infiltration of inflammatory cells), necrosis (morphologic changes of renal parenchymal cells consistent with degeneration and necrosis) and vascular disruptions (hemorrhage, edema and other signs of vascular leakage). Score “0” means unremarkable lesions and score “1” means minimal lesions. Specific lesions in the samples with minimal lesions (score =1) included small focus of regenerative tubules in saline treated mice while in the doxycycline-gentamicin treated mice showed large focus of perivascular mononuclear cells at the renal pelvis.

Table 2.2. 3: Peri-renal adipose histopathological scores of hematoxylin and eosin and Perl's Prussian Blue stained sections of kidneys from MBICs treated mice.

Kidney number	Saline (Peri-renal adipose)		Doxycycline-Gentamicin (Peri-renal adipose)		Doxycycline-MBICs (Peri-renal adipose)	
	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2
1	0	0	1	1	1	1
2	0	0	1	1	0	0
3	0	0	0	0	0	0
4	0	0	0	1	1	1
5	0	0	1	1	1	1
6	0	0	0	0	1	1
7	0	0	0	0	1	1
8	0	0	0	0	0	0

Perirenal adipose of kidneys was examined by two pathologists and scored on the basis of lesions present. Lesions included inflammation (infiltration of inflammatory cells), necrosis (morphologic changes of renal parenchymal cells consistent with degeneration and necrosis) and vascular disruptions (hemorrhage, edema and other signs of vascular leakage). Score "0" means unremarkable lesions and score "1" means minimal lesions. Specific lesions (scored 1) included the small focus of mononuclear cells in saline and doxycycline-gentamicin treated mice while in doxycycline-MBICs treated mice it represent the aggregates of pigment laden macrophages admixed with mononuclear cells.

Chapter 2.3

In vivo efficacy of gentamicin loaded magnetite block ionomer clusters (MBIClusters) against *Brucella melitensis*

In the last chapter we have shown that compared to free gentamicin, gentamicin loaded magnetite block ionomer complexes (MBICs) were very effective against *Brucella melitensis in vitro* but failed to show higher efficacy *in vivo*. One of the major speculations was the small size of MBICs (64 nm) that might have shortened the circulation time in the mice. In the present work we aimed to increase the size of MBICs by attaching MBICs molecules to generate clusters of MBICs (MBIClusters) and test the efficacy to kill *B. melitensis* in infected mice.

To synthesize MBICs clusters, first, PEO-*b*-PAA-NH_2 polymer was synthesized as described in chapter 2.2. MBIClusters were generated according to the scheme shown in Figure 2.3.1. MBICs were synthesized as described in chapter 2.2 but instead of using PEO-*b*-PAA, amine functionalized PEO-*b*-PAA (PEO-*b*-PAA-NH2) was used. The amine termini on the tips of the PEO shells of MBICs were cross-linked through an azo-Michael reaction to form small MBIClusters. The MBICs were dispersed in DI water at the prescribed concentration (10 mg/ml, 20 mg/ml or 30 mg/ml) and sonicated for five min. A 700 g mol^{-1} PEG diacrylate was dissolved in a separate vial at the same concentration in DI water. With stirring, a stoichiometric amount of the PEG diacrylate solution was added dropwise into the MBIC dispersion over approx. two min., then the pH was

adjusted to 7 with 1 N NaOH. The dispersion was stirred at 37 °C for 24 hr, then dialyzed against DI water for 24 hr to remove any unreacted diacrylate. The product was recovered by freeze-drying. Gentamicin loaded MBIClusters were synthesized by the same procedure described in chapter 2.2 to synthesize gentamicin loaded MBICs. Amount of gentamicin loaded in MBIClusters was determined using *o*-phthalaldehyde assay as previously described (1). The size and zeta potential of MBICs were characterized by DLS with a Malvern Zetasizer Nano ZS. The efficacy of MBIClusters was tested to mice to clear *B. melitensis* infection and was compared to free gentamicin. Gentamicin, either free or MBIClusters was administered in combination of doxycycline as we have shown before that combination is more efficacious than monotherapy to treat *B. melitensis* infection in mice. Almost 6-8 weeks old BABL/c mice were infected with 5 x 10^4 *B. melitensis* i.p. After 6 weeks of infection, mice were treated with doxycycline (3 mg/kg body wt., i.p.) and gentamicin (5 mg /kg body wt., i.p.). Gentamicin was either given as free gentamicin or loaded in MBIClusters. Mice were treated every day for 28 days and were euthanized 48 hr after the end of treatment. Spleens and livers were collected from mice to determine *Brucella* CFUs. Kidneys were then fixed in 10% neutral buffered formalin, routinely processed, embedded into paraffin blocks, and 5 μm sections were stained with hematoxylin and eosin on glass slides (Virginia-Maryland Regional College of Veterinary Medicine, Veterinary Teaching Hospital).

Compared to MBICs, MBIClusters were larger in size and showed intensity average diameters of 152 nm. After encapsulating the gentamicin, the intensity

average diameter reduced to 109 nm because of the collapse of complexes caused by the electrostatic neutralization by gentamicin. MBIClusters showed a zeta potential of -60 mV that was increased to -20mV after encapsulation of gentamicin. Table 2.3.1 shows the *Brucella* CFUs in spleens and livers of mice received different treatments. Although, there is a statistically significant difference in the mice that received antibiotics from the mice that were treated with saline but there was no difference between that mice treated with doxycycline in combination with either free gentamicin or gentamicin loaded MBIClusters. Histopathological examination shows the similar deposition of macrophages containing MBIClusters in perirenal adipose (Figure 2.3.2) as seen previously in MBICs treated mice.

Thus, it can be concluded that increasing the size of MBICs did not enhance the *in vivo* efficacy against *B. melitensis*. We speculate that this is because of the uptake of MBICs or MBIClusters by circulating phagocytic cells and inability to reach the targeted organs like spleens and livers. Increase in size is not enough to address this problem. Further studies are required to understand the *in vivo* distribution of MBICs in mice. Magnetite nanoparticles that makes the core of both MBICs and MBIClusters are powerful contrast enhancement agents for T2-weighted magnetic resonance imaging (MRI) because of their high magnetization, low toxicities and particulate nature (2, 3). Thus, magnetite imaging resonance (MRI) techniques should be used to study the *in vivo* distribution of MBICs or MBIClusters in mice.

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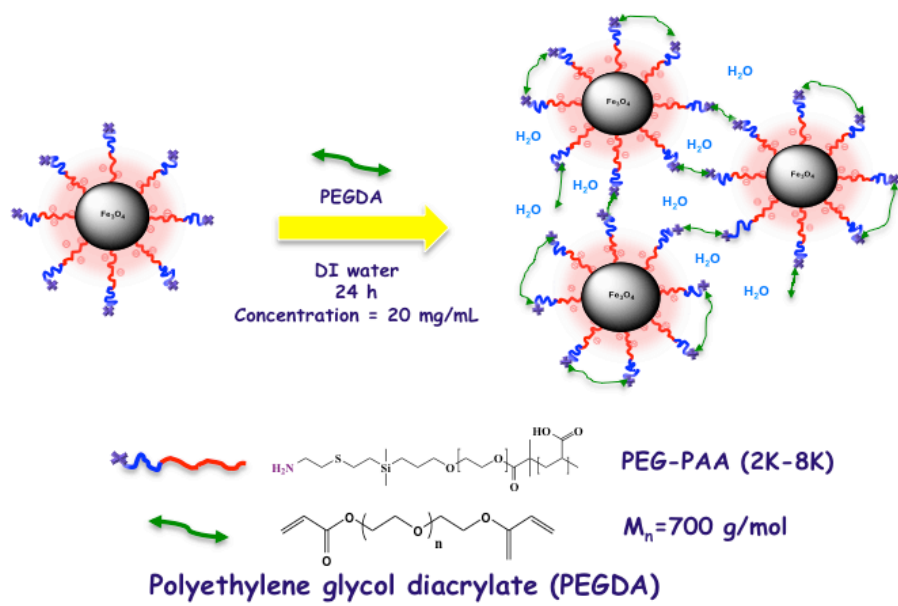

Figure 2.3.1: Scheme to synthesize MBIClusters from MBICs.

MBICs are fitted with reactive amine termini on the outer layer of the shells that can be cross-linked to provide controlled size clusters.

Table 2.3. 1*: In vivo* efficacy of gentamicin loaded MBIClusters in combination with doxycycline to treat *B. melitensis* infection in mice.

Treatment group	CFUs (Log_{10}) in spleens	CFUs (Log_{10}) in livers
Saline	5.1± 0.46	3.3 ± 0.62
Doxycycline + MBIClusters	2.7 ± 0.08*	1.5 ± 0.17*
Doxycycline + Gentamicin	2.6 ± 0.13*	1.5 ± 0.22*

Mice were infected with *B. melitensis* and after 6 weeks of infection, treated with either doxycycline + gentamicin loaded MBIClusters or doxycycline + free gentamicin. All the drugs were administered i.p., mice were treated once daily for 28 doses. Saline treated mice were the negative controls. The data present here is the mean ± SEM of 5 mice and asterisk (*) represents the statistically significant ($p \leq 0.001$) from the negative controls.

Table 2.3. 2: Renal histopathological scores on examination of hematoxylin and eosin (H&E) stained sections of kidneys from MBIClusters treated mice.

	Saline (Kidney score)		Doxycycline-Gentamicin (Kidney score)		Doxycycline-MBIClusters (Kidney score)	
Kidney no.	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	2	2	0	0
6	0	0	1	0	1	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0

Kidneys were examined by two pathologists and scored on the basis of lesions present. Lesions included inflammation (infiltration of inflammatory cells), necrosis (morphologic changes of renal parenchymal cells consistent with degeneration and necrosis) and vascular disruptions (hemorrhage, edema and other signs of vascular leakage). Score 0 = unremarkable lesions, 1 = minimum lesions and 2 = mild lesions. Specific lesions were as follows: Group-doxycycline-gentamicin, kidney # 5 (scored “2”) showed suppurative pyelonephritis.

Table 2.3. 3: Peri-renal adipose histopathological scores of hematoxylin and eosin and Perl's Prussian Blue stained sections of kidney obtained from MBIClusters treated mice.

	Saline (Peri-renal adipose)		Doxycycline-Gentamicin (Peri-renal adipose)		Doxycycline-MBIClusters (Peri-renal adipose)	
Kidney no.	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2	Pathologist 1	Pathologist 2
1	0	0	0	1	2	2
2	0	0	0	1	1	1
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	1
6	0	0	0	0	1	1
7	0	0	0	0	1	1
8	0	0	0	0	1	1

Perirenal adipose of kidneys was examined by two pathologists and scored on the basis of lesions present. Lesions included inflammation (infiltration of inflammatory cells), necrosis (morphologic changes of renal parenchymal cells consistent with degeneration and necrosis) and vascular disruptions (hemorrhage, edema and other signs of vascular leakage). Score 0 = unremarkable lesions, 1 = minimum lesions and 2 = mild lesions. Specific lesions included aggregates of pigment laden macrophages admixed with mononuclear cells in mice treated doxycycline-MBIClusters

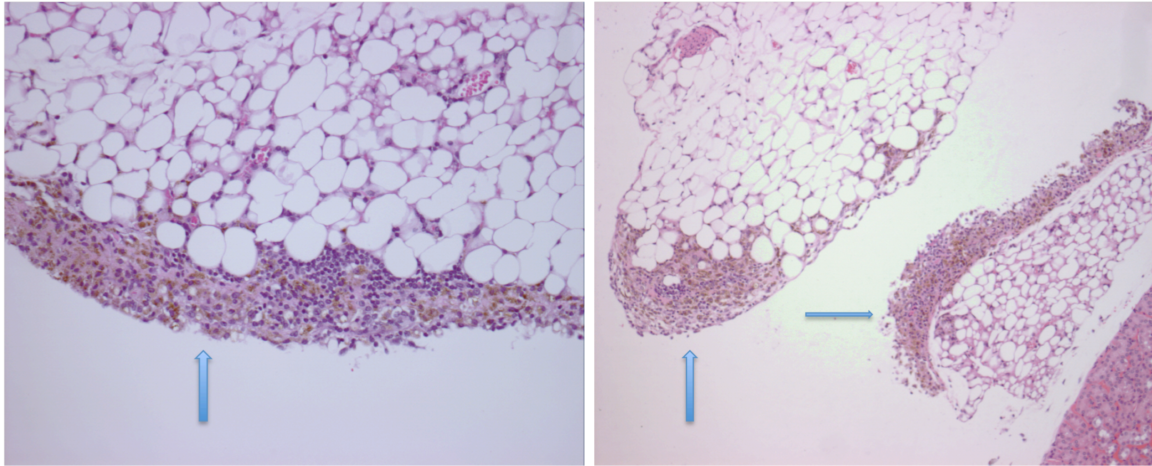

Figure 2.3.2: Hematoxylin and Eosin (H&E) stained kidney section from the mice treated with MBIClusters.

Arrows show the deposition of brown pigment laden macrophages in peri-renal adipose and infiltration of mononuclear cells.

Chapter 3

Pluronics P85 enhances the efficacy of outer membrane vesicles as a subunit vaccine against *Brucella melitensis* challenge in mice

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Abstract

In spite of brucellosis being the most common zoonotic disease worldwide but still there is no vaccine for human use. *Brucella melitensis* is the most pathogenic species of *Brucella* to humans, and it is a threat to be used as bio-weapon. *B. melitensis* Rev1, a live-attenuated strain, is the commercial vaccine for small ruminants to prevent *B. melitensis* infections but has been associated with abortions in animals. Because of its side effects it cannot be used to vaccinate human beings. Outer membrane vesicles (OMVs) obtained from *B. melitensis* have been shown to provide protection similar to strain Rev1 in a mouse model against *B. melitensis* challenge. In the present work we tested the efficacy of Pluronic P85 to enhance the efficacy of (OMVs) as a vaccine. P85 enhanced the *in vitro* secretion of TNF-α by macrophages incubated with OMVs and P85. Further, P85 enhanced the protection provided by OMVs against *B. melitensis* challenge. This enhanced protection was associated with higher total IgG antibody production but not increased IFN-γ or IL-4 cytokine levels. Moreover, P85 alone provided the significantly better clearance of *B. melitensis* compared to saline

vaccinated mice. Further studies are warranted to find the mechanism of action of P85 that provided non-specific protection and enhanced the efficacy of OMVs as a vaccine against *B. melitensis*.

Introduction

Brucellosis, caused by *Brucella* species, is the most common zoonotic disease worldwide (Corbel, 1997). *Brucella* is a Gram negative, facultative intracellular pathogen that causes infection in almost all domestic species of animals and humans. *B. melitensis*, *B. suis* and *B. abortus* are most pathogenic to humans. Because of its potential to be easily aerosolized, *Brucella* is an ideal bioterrorism agent and thus classified as class B agent by Center for Disease Control and Prevention (CDC) (Pappas*, et al.*, 2005). Through massive eradication programs in animals, the disease has been eradicated from many developed countries but is still endemic in Latin America, Middle East, Spain, parts of Africa and Western Asia (Memish & Balkhy, 2004). It is not possible to implement eradication plans in these parts of the world because of economical, social and religious reasons. Thus, infected animals and their products remain as reservoirs for human infection. Although *Brucella* infection is rarely fatal in humans, it is severely debilitating and disabling (Perkins*, et al.*, 2010). At present there is no vaccine available for human use. Live attenuated, smooth *B. melitensis* strain Rev1 is widely used to vaccinate small ruminants to protect against *B. melitensis.* However, it has been shown to cause abortions in pregnant ruminants and is not considered safe for human use. More than 500,000 new cases of human brucellosis occur every year, thus there is an urgent need for a vaccine to protect humans.

Because *Brucella* is an intracellular pathogen, cell mediated immunity (CMI) plays the central role in acquired resistance by the host against brucellosis (Baldwin & Goenka, 2006). CMI to intracellular pathogens is generally characterized by IFN-γ produced by CD4+ Th1 and CD8+ Tc1 cells to limit intracellular *Brucella* survival and replication, CD8+ cytotoxic T cells to kill infected macrophage and Th1 associated antibodies to enhance opsonization and phagocytosis of *Brucella*. A live attenuated strain as a vaccine is usually considered the best vaccine candidate against intracellular pathogens, as it expresses *in vivo* antigens and thus stimulates the required immune response. So far no live attenuated strains of *Brucella* have been shown to be safe enough to be used in humans.

Subunits vaccines such as recombinant proteins and synthetic peptides are safer than live attenuated vaccines but often require an adjuvant and boosters as many antigens are weak immunogens (Byars & Allison, 1987). Adjuvants enhance the potency of subunit vaccines either by providing a depot for the vaccine for slow release, targeting the antigen to the immune cells or by modulating and enhancing the immune responses either towards a Th1 or Th2 type (Mallapragada & Narasimhan, 2008). Currently, aluminum compounds are the only adjuvants approved for human use in United States of America (U.S.A.). Although the aluminum compounds are safe to use, they are very weak adjuvants and often require multiple doses to elicit the full response of the vaccine. Many new adjuvants are under investigation but are restricted either because of toxic effects or the need for sophisticated techniques to incorporate antigens. Some immunomodulators like LPS derivatives, cytokines, oligonucleotides containing CpG motifs etc. have been tested as adjuvants as well (Coffman, *et al.*, 2010). Interestingly,

synthetic polymers elicit immunostimulation and show a great potential to be used as immunostimulatory vaccine adjuvants. Many studies have shown the potential use of Pluronics as an adjuvant to increase both cell mediated and antibody mediated immune responses elicited when used with broad spectrum of antigens (Hunter*, et al.*, 1981, Snippe*, et al.*, 1981, Allison & Byars, 1986). Pluronics or Polaxamers are non-ionic block copolymers of poly(propylene oxide) (PPO) and poly(eythylene oxide) (PEO) (Batrakova & Kabanov, 2008) and have been shown to activate pro-inflammatory signaling pathways and immune responses. Moreover, the formulations only require the mixing of antigens with pluronics at an appropriate concentration. Pluronic P85 is ampiphilic in nature and unlike hydrophobic Pluronics like L61 are found to be non-toxic to the injected muscles (Gaymalov*, et al.*, 2009). P85 enhances the systemic and local expansion of antigen presenting cells (APCs) that includes dendritic cells (DCs), macrophages and natural killer cells.

Recently we have shown the potential of outer membrane vesicles (OMVs) obtained from *B. melitensis* 16M to protect against *B. melitensis* infection in mice (Avila-Calderon*, et al.*, 2012). OMVs are double membrane structures that are naturally released by Gram-negative bacteria (Beveridge, 1999). OMVs are mainly comprised of the outer membrane, periplasmic proteins, LPS and inner membrane (Kuehn & Kesty, 2005). Mice vaccinated with OMVs with incomplete Freund's adjuvant (IFA) showed similar protection as provided by the strain Rev1 vaccine in mice against *B. melitensis* challenge. But IFA is not approved for human use in United States. In the present study, the aim was to explore the potential of synthetic Pluronics as adjuvant to enhance the efficacy of OMVs as a vaccine candidate for human use in the future.

Materials and Methods

Bacterial strains, cell lines: Wild type *B. melitensis* 16M was from our culture collection at Virginia Tech, Blacksburg, VA. *Brucella* was regularly grown on tryptic soy agar (TSA) and in tryptic soy broth (TSB) at 37°C in a 5% CO_2 environment. For *in vitro* studies, J774A.1 murine macrophages like cell line (ATCC) was used. J774A.1 cells were regularly grown in Dulbecco's modified Eagle's media (DMEM, Sigma-Aldrich Inc.) containing 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Penstrep, Cellgro)

Purification and Characterization of OMVs: OMVs were obtained from *B. melitensis* 16M as previously described (Gamazo*, et al.*, 1989) but with the following modifications. Briefly, stock cultures of *B. melitensis* 16M were streaked on TSA plates and incubated for 48 hr. Using cotton swabs, the bacterial culture was transferred to TSA slants containing 0.7% yeast extract and allowed to grow for 48 hr. Next, the bacterial culture was scraped from slants using TSB (1 mL/slant) and collected and spread on TSA plates (150 mm x 15 mm, 100 plates) containing 0.7% yeast extract. After an incubation of 48 hr, bacteria were with 200 µL PBS/plate using a sterile cell scrapper. The bacterial suspension was centrifuged at 15,191 x g for 30 min and supernatant was collected. The supernatant was filter sterilized twice using 0.22 µm filters and the sterility of the filtrate was tested by inoculating 50 µL into 10 mL TSB and incubating at 37°C for 48-72 hr. Absence of any bacterial growth confirmed the sterility of the filtrate. To obtain the OMVs, the filtrate was ultracentrifuged at 176,508 x g for 2 hr. The pellet was washed in

50 ml PBS and harvested by centrifugation. The washing step was repeated twice and finally the pellet containing OMVs was resuspended in 250 µL PBS and stored at -20°C. Concentration of protein in OMVs was determined using Pierce BCA protein Assay kit (Thermo Scientific) according to the manufacturer's protocol. For the assessment of structure of OMVs using by electron microscopy, OMVs were mixed with 2% aqueous uranyl acetate solution for negative staining and placed onto 200-mesh formvar, carbon coated copper grids (Electron Microscopy Sciences). Excess liquid was soaked away using filter paper and the samples were viewed at 63,000x or 100,000x magnification on a Zeiss 10CA Transmission Electron Microscope (Virginia-Maryland Regional College of Veterinary Medicine). To determine the protein profile, first OMVs were mixed with different concentrations of P85, dissolved in phosphate buffered saline, using a double hub-syringe and then mixed with 2X Laemmli buffer (Bio-Rad) containing β-mercaptoethanol and heated for 5 min at 95°C. The samples were electrophoresed on 10% SDS-PAGE gels (Invitrogen) and stained with Coomassie blue to visualize protein bands.

In vitro Studies: 1) Cytotoxicity of P85: Cell titer 96® Aqueous Non-Radiocative Cell Proliferation MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay Solution (Promega) was used to determine any possibility of cell cytotoxicity caused by P85 dissolved in saline. Briefly, ~2 x 10^5 J774A.1 cells/well, suspended in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin, were seeded in 96-well tissue culture plates (Corning Inc.). After 24 hr of incubation, the media was removed and a series of concentrations of P85 were added

to cells along with the DMEM containing 10% FBS and 1% penstrep and incubated for 24 hr. The cells were washed with PBS twice and then 100 µL/well of fresh media was added to the cells. Cell Titer 96® Aqueous reagent solution (20 µL) was added to each well except for those containing the negative controls. The cells were incubated in the dark for 2-3 hr at 37°C in a humidified 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using a 96-well ELISA plate reader. The percentage cell viability was calculated considering the OD values for the control wells as 100% cell viability.

2) Quantification of TNF-α secreted by J774A.1 macrophages:

Approximately, 5×10^5 J774A.1 cells were seeded per well in a 96 well plate in DMEM + 10% FBS + 1% penstrep. After 24 hr, cells were washed with PBS and media containing either OMVs (2 µg/well) alone or OMVs with different concentrations of P85 were added to the cells in triplicates. Medium was added as a negative control while *E. coli* LPS (1 µg/well) was added as positive control. After an incubation for 24 hr, culture medium was collected and levels of TNF-α were determined using Mouse TNF ELISA Set II (BD pharmingen) according to the manufacturer's protocol. Also the cell cytotoxicity was tested as described before to confirm the cell viability after incubation with OMVs and P85.

In vivo studies: 1) Mice vaccination and challenge: A total of 30 mice (5 mice each group) were vaccinated for the first phase of the study with OMVs (5 µg/ mouse) alone or with different adjuvants (IFA, 0.3% P85, 0.03% P85, 0.003% P85) given intramuscular. OMVs were mixed with the adjuvants using a double hub needles with two syringes and the volumes were adjusted with PBS. After 3 weeks all the mice were

given a booster equal to the primary dose. One week after boost dose all the mice were bled retro-orbitally. The plasma was collected and used to determine antibodies titers (described later). After one more week mice were challenged with 2 x 10^4 CFUs/mouse of *B. melitensis* 16M given i.p. After 2 weeks post-challenge, mice were euthanized and spleens were collected. Spleens were homogenized in 2 mL TSB and serial dilutions of homogenates were prepared in TSB and were plated on TSA plates. The plates were incubated for 72 hr under 5% CO_2 and bacterial CFUs were determined.

In the second phase of the study, a total of 32 mice (8 mice per group) were vaccinated either with saline, 0.03% P85, OMVs or OMVs + 0.03% P85. Mice were boosted with the same dose 3 weeks later. After two weeks, each group was divided into two sets. The first set of mice (5 mice each) were challenged with *B. melitensis* and euthanized 2 weeks after challenge to determine bacterial loads in spleens as described before. The second set of mice (3 mice each) was used to obtain splenocytes and to perform lymphocyte proliferation assays and to determine cytokines in culture supernatants (described below).

2) Splenocyte culture and quantification of IL-4 and IFN-γ: Splenocytes from the vaccinated mice (3 mice per group) were obtained as previously described (Vemulapalli, *et al.*, 1998). Approximately, 5 x 10^5 splenocytes/well were seeded in flat bottom 96 well plates and stimulated with different concentrations of OMVs obtained from *B. melitensis* 16M. Splenocytes were stimulated with media alone as a negative control and with Concavalin A (1 μg/mL) as a positive control. After incubating cells for 5 days at 37°C, the supernatants were collected and concentrations of IL-4 and IFN-γ were determined using cytokine ELISA kit (BD Pharmingen) according to the manufacturer’s protocol.

3) Antibody titers in plasma: An indirect ELISA was performed to measure the levels of *B. melitensis* specific antibodies in the plasma. Briefly, heat inactivated *B. melitensis* 16M (stock culture was heated at 80°C for 1 hr, inactivation was confirmed by sterility of the culture after 48 hr of incubation) was adsorbed to wells of polystyrene plates (Nunc Maxisorp) at the protein concentration of 1.0 µg/mL in 50 µl of bicarbonate buffer (pH 9.6). After incubating overnight at 4°C, plates were washed 4 times with phosphate buffer saline (PBS) containing 0.05% Tween-20. The wells were then blocked with 2% bovine serum albumin in PBS and incubated for 2 hr at room temperature. The plates were washed 4 times as before. Mice plasma diluted at 1:6400 was added in duplicates to the wells and incubated for 3 hr at room temperature. Again, the plates were washed 4 times and isotype specific goat anti-mouse horseradish peroxidase conjugates was added for 30 min at room temperature. After washing the plates 4 times, 100 µL of TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated in dark for 20 min. The reaction was stopped by adding 100 µL/well of 0.18 M sulfuric acid and the absorbance of the developed color was measured at 450 nm.

<u>Statistical analysis:</u> All the statistical analysis was done using Microsoft EXCEL. A two-tailed Student *t*-test (Microsoft EXCEL) was used to determine *p*-values.

Results and Discussion

<u>Characterization of OMVs:</u> Electron microscope images of negatively stained OMVs with uranyl acetate (Figure 3.1) show the characteristic double membrane structure of OMVs. The size ranged between 50-120 nm. Commassie blue stained SDS PAGE images revealed the similar protein patterns of OMVs obtained from *B. melitensis* 16M

shown previously. A range of protein bands was present with major bands between 25-30 kDa that agrees with data shown previously (Gamazo & Moriyon, 1987). Mixing with different concentrations of P85 did not change the protein profile of OMVs (Figure 3.2) on SDS PAGE gels. This suggests that proteins in OMVs were not modified after mixing with P85 as it has surfactant like properties. Moreover, the mild detergent like properties might have contributed to the stabilization of proteins of OMVs by preventing the aggregation of proteins as previously demonstrated for F127 pluronic (Wang & Johnston, 1993, Katakam & Banga, 1997). Further studies are required to examine the physical interaction between OMVs and P85.

Toxicity of P85 and *in vitro* secretion of TNF-α by macrophages: As mentioned before, Pluronic P85 is ampiphilic and displays surfactant properties including interactions with hydrophobic surfaces and biological membranes. These interactions could be toxic to the cells when pluronics are used at a high concentration. Moreover, it has been shown that Pluronics reduce the ATP synthesis by cells and affect cell viability when used at high concentrations (Batrakova & Kabanov, 2008). We choose to test the concentration equivalent to the critical micellar concentration (CMC) of P85, 0.03%, (Kabanov*, et al.*, 2000), and one log higher and one log lower than the CMC, defined as the concentration of the block polymer at which micelles are formed (Kabanov & Alakhov, 2002). Usually below the CMC the non-ionic block co-polymers exist in the form of unimers. Above the CMC, there is equilibrium between unimers and micelles. Depending upon the method used to determine CMC of Pluronics, it is accepted that CMC will vary from 3 to 10 times. Our results show that P85 causes a concentration

dependent toxicity to the macrophages. At the concentration of 0.3%, P85 was found to be highly toxic to the macrophages (Figure 3.3). When added at the CMC (0.03%) or lesser concentrations to the macrophages, P85 remained nontoxic. For further *in vitro* experiments, P85 was used at the concentration of 0.03% or less. For the *in vivo* experiments, P85 was tested up to the concentration of 0.3% as it was shown to be nontoxic by others (Gaymalov*, et al.*, 2009).

Culture supernatants of J774A.1 cells stimulated with either OMVs or OMVs + P85 show induction of TNF-α (Figure 3.4), a proinflammatory cytokine and an indicator of stimulation. Compared to OMVs alone, a mixture of OMVs and P85 (0.03%) significantly increased secretion of TNF-α by the macrophages. This increase was P85 concentration dependent, and the highest secretion was seen at the concentration of 0.03 % of P85 mixed with OMVs. Although OMVs contain LPS, *Brucella* LPS is not as strong an immunostimulator as *E. coli* LPS (Lapaque*, et al.*, 2006). The presence of outer membrane protein 25 (omp25) in *B. suis* (that is also present in OMVs) has been shown to be associated with down-regulation of TNF-α secretion by human macrophages (Jubier-Maurin*, et al.*, 2001). Therefore, it was no surprise to find lower levels of TNF-α secreted by macrophages stimulated with OMVs. The addition of 0.003% P85 to OMVs also resulted in higher levels of TNF-α secreted by macrophages. There was no effect of P85 alone at two different concentrations on the secretion of TNF-α. Similar stimulatory effects on mouse alveolar macrophages cells (RAW 264.7) were induced by hydrophilic Pluronic F127, when used along with chitosan to prepare microspheres to encapsulate *Bordetella bronchoseptica* multiple antigens (BBD) (Kang*, et al.*, 2007). But concentration-dependent stimulation was not shown for Pluronic F127.

Our results show that the stimulatory effect of OMVs on macrophages can be increased by the addition of P85 at a specific concentration without causing any toxicity to the cells.

Protection in mice against *B. melitensis* challenge: First we determined the efficacy and appropriate concentration of P85 to enhance the potential of OMVs as a vaccine candidate against *B. melitensis* challenge. Table 3.1 shows that mice vaccinated with OMVs + 0.03% P85 showed better protection against *B. melitensis* compared to OMVs alone or OMVs+ IFA. P85 worked best at the CMC but not a log higher or lower in combination with OMVs. Although, it has been shown before that P85 enhances the subunit or DNA vaccine efficacies but not many research studies included a range of Pluronics concentrations, which from our study appear to be an important factor to consider.

In the second phase we determined the level of protection provided by OMV+P85 in mice against *B. melitensis* challenge. To our surprise, 0.03% P85 provided significantly higher level of protection in mice compared to the mice vaccinated with saline. Previously it has been shown that poly (anhydride) polymers alone were able to induce Th1 responses in mice and provided significant protection in mice after challenge with *Salmonella enterica* (Tamayo*, et al.*, 2010). Polyanhydride polymers were shown to act as agonists to various toll like receptors (TLR 2, 4 and 5) and to trigger the secretion of Th1 profile cytokines like IFN-γ and IL-12. Neudeck *el. al*. showed that administration of P85 alone resulted in reduced loads of bacteria in spleens and livers of the mice challenged with *Listeria monocytogenes* (Neudeck*, et al.*, 2008). Depletion of ATP by

P85 was speculated as the reason for this finding, but it was not proven in a mouse model. Another Pluronic, CRL-1072 was shown multiple times to kill *Mycobacterium tuberculosis* in cell cultures as well as in mice but no satisfactory explanation was provided for this activity (Jagannath*, et al.*, 2000). Moreover, *in vitro* toxicity by CRL-1072 was not reported and this might be the reason for the elimination of *Mycobacterium* from the cells. It was speculated that the surfactant like property of Pluronics helps in disrupting the lipid membrane of *Mycobacterium* spp. and thus helps in clearing the pathogen from infected mice (Jagannath*, et al.*, 1999). Thus, it can be said that Pluronics are capable of enhancing clearance of intracellular pathogens (*Mycobacterium* spp. and *Brucella* spp.) in mice when given either before or after challenge. In our study, mice were challenged with *Brucella* after 2 weeks of administration of P85 and thus it is unlikely that P85 interacted with *Brucella* to disrupt their cell membranes. Thus, further studies are required to understand the mechanism by which Pluronic alone can provide significant protection when given before challenge with different bacterial species in mouse model.

Cytokine secretion by splenocytes: Levels of IFN-γ and IL-4 cytokines were determined in the culture supernatant from stimulated splenocytes obtained from mice vaccinated with saline, P85, OMVs or OMVs + P85. High levels of IFN-γ were measured in the splenocytes culture supernatant from OMVs or OMVs + P85 vaccinated mice upon stimulation with OMVs at different concentrations (Figure 3.5). Levels of IL-4 were below the detection limit in all the samples. This shows the presence of a stronger Th1 stimulation by OMVs compared to the Th2 type immune response as speculated by us

before (Avila-Calderon*, et al.*, 2012). There was no significant differences in the levels of IFN-γ in OMVs vs. OMVs + P85 vaccinated mice which again indicate some other mechanism by which P85 is able to enhance the protection provided by OMVs. Levels of IFN-γ detected in mice vaccinated with P85 vs. saline vaccinated mice upon stimulated with OMVs shows the absence of non-specific Th1 stimulation of cytokines in P85 vaccinated mice.

<u>Antibody titers in plasma:</u> In general, cell mediated immune responses are required to control intracellular pathogens. However, it has been shown that both arms of immune system, Th1 and Th2, work together to provide protection against *B. melitensis* challenge (Delpino*, et al.*, 2007, Moustafa*, et al.*, 2011). Presence of antigen specific antibodies in the serum show the activation of both Th1 and Th2 immune responses in the mice vaccinated with OMVs. The levels of total IgG were significantly higher in the mice vaccinated with OMVs + P85 compared to mice receiving only OMVs (Figure 3.6). Other pluronics like F127 have been shown to enhance the antibody titers when used with antigens as adjuvant (Kang*, et al.*, 2007). IgG subtypes, IgG1 and IgG2a are indicative of Th2 and Th1 responses respectively but there were no significant differences in either isotype in mice vaccinated with OMVs vs. OMVs + P85. Antibodies have been shown before to provide protection against *B. melitensis* infection in mouse model (Montaraz*, et al.*, 1986, Limet*, et al.*, 1987). Increased levels of IgG might have helped in clearance of circulating *Brucella* and enhanced the protection against *B. melitensis* challenge in OMVs+P85 vaccinated mice.

In conclusion, we demonstrated that Pluronic P85 enhances the efficacy of OMVs obtained from *B. melitensis* as a vaccine candidate against *B. melitensis* challenge in a mouse model. The better protection provided by OMVs+P85 may be due in part to the induction of higher antibodies production in mice. Further, it might be the additive effect of antigen specific protection provided by OMVs and non-specific protection provided by P85. The mechanisms by which P85 enhanced the efficacy of OMVs and provided protection alone are still not clear and further experiments are needed.

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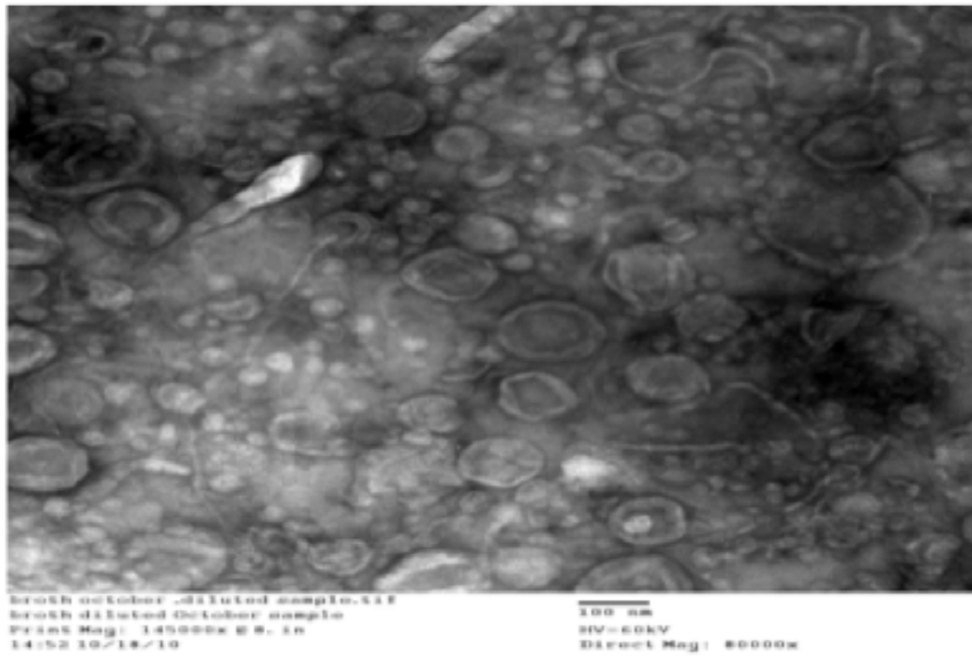

Figure 3.1: Electron microscope image of OMVs obtained from *B. melitensis* 16M. OMVs visualized at 60000 x magnification showing the double membraned, outer membrane vesicles obtained from *B. melitensis* 16M. The size ranges between 50-100 nm

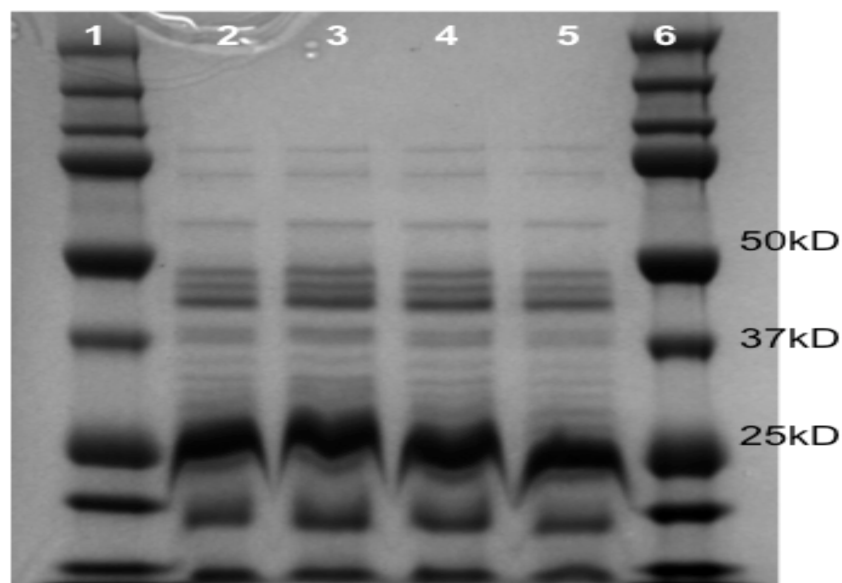

Figure 3.2: Coomassie blue stained 10% SDS-GEL picture showing the protein profile of OMVs and OMVs mixed with different concentrations of P85.

In all the samples concentration of OMVs was approximately 5µg. Lane 1 and 6: Standard protein ladder, Lane 2: OMVs, Lane 3: OMVs + 0.003% P85, Lane 4: OMVs+ 0.03% P85, Lane 5: OMVs + 0.3% P85.

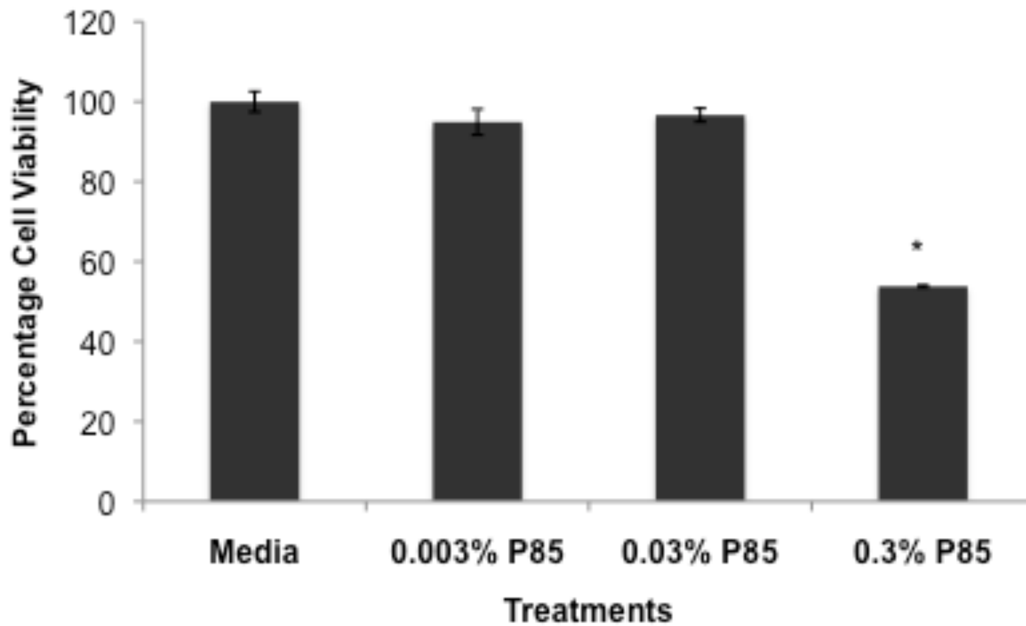

Figure 3.3: Effect of addition of P85 at different concentrations on the viability of J774A.1 cells.

Cells were incubated with different concentrations of P85 and cell viability was determined using MTS assay after 24 hr of incubation. The cell viability was determined considering the viability of cells incubated with media only as 100%. Data presented here is the mean ± SEM of cell viability obtained from three independent experiments and asterisk (*) represents statistical significant difference at $p \leq 0.001$.

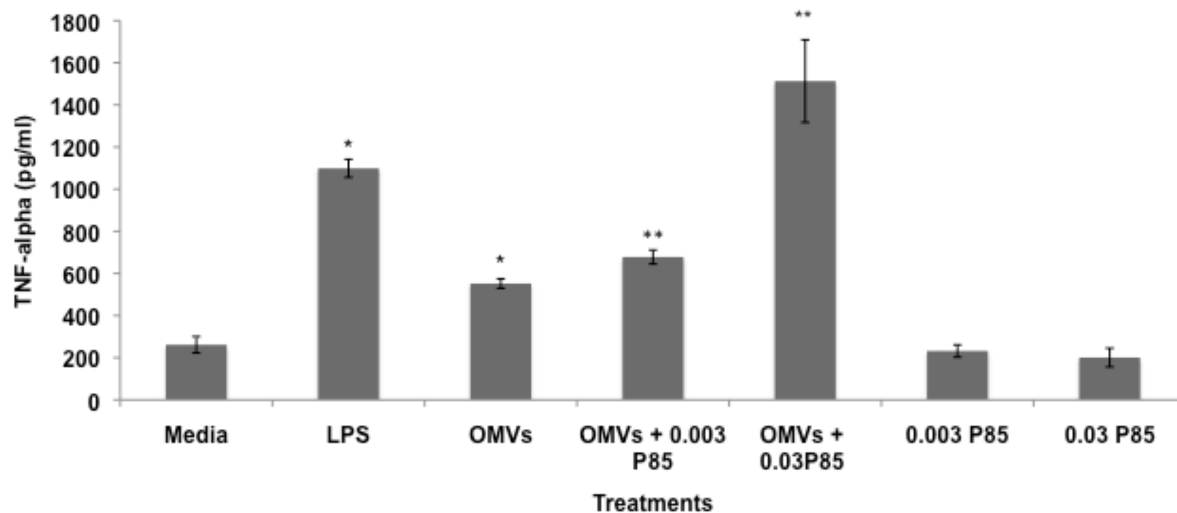

Figure 3.4: Production of TNF-α by J774A.1 macrophages incubated with OMVs and P85.

P85 mixed with outer membrane vesicles (OMVs) obtained from *B. melitensis* 16M induces higher level of TNF-α secretion from J774A.1 cells than induced by OMVs alone. To assess the stimulation of J774A.1 cells different concentrations of P85 mixed with OMVs or OMVs alone or P85 alone were added to the cells. After 24 hr of incubation, levels of TNF-α were determined in culture supernatant using indirect sandwich ELISA. Media and *E. coli* LPS treated cell supernatants served as negative and positive controls respectively. Lower limit of detection was 15 pg/ml. Levels of TNF-α are shown as mean ± SEM of three independent experiments. One asterisk (*) represents the statistically significant difference at $p \leq 0.001$ from media and two asterisks (**) represent statistically significant difference at $p \leq 0.001$ from media as well as from OMVs alone.

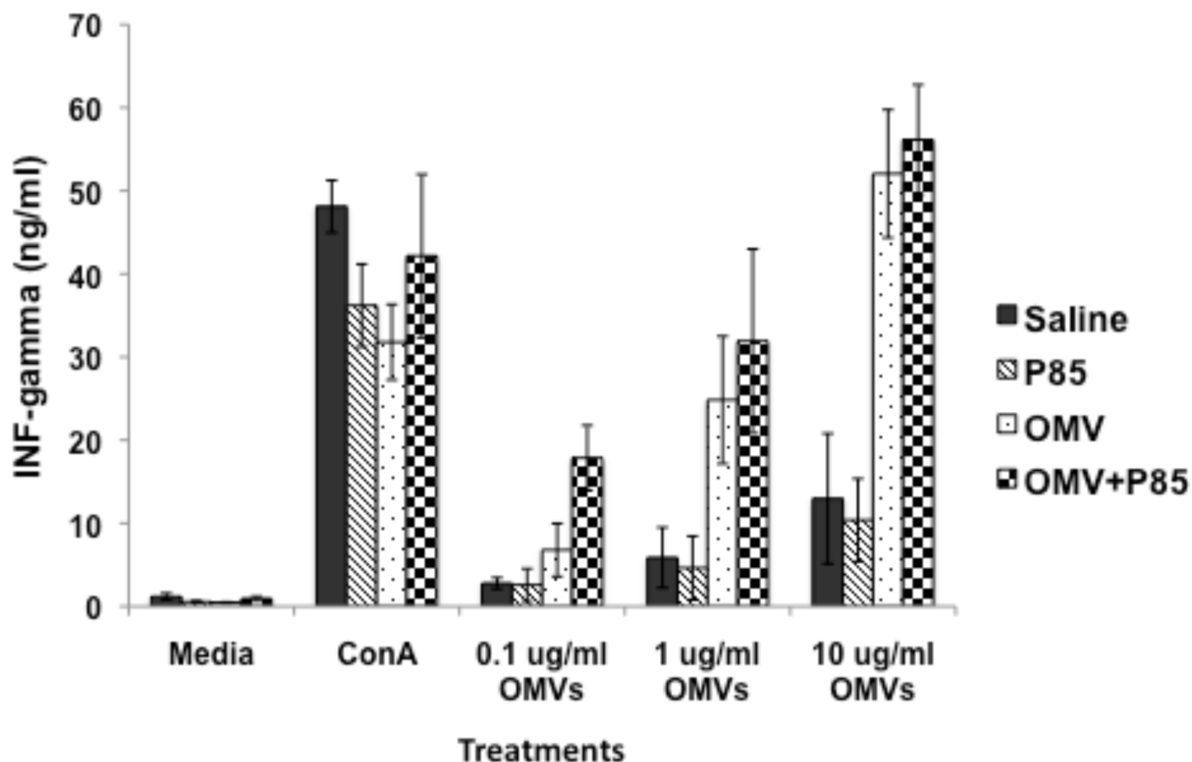

Figure 3.5: Production of IFN-γ by splenocytes obtained from vaccinated mice.

IFN-γ secretion by splenocytes obtained from mice vaccinated with saline, P85, OMVs or OMVs + P85 upon treatment with different concentrations of OMVs. IFN-γ levels were determined in splenocyte culture supernatants 5 days after the stimulation using indirect sandwich ELISA. Splenoctye supernatants from media and ConA treated cells were negative and positive controls respectively. Higher and lower limits of detection of IFN-γ were 200 ng/mL and 1.6 ng/mL respectively. Data represent results from 3 mice per group and 3 independent samples per mouse. The graph shows the mean ± SEM of IFN-γ levels. Asterisk (*) shows the statistically significant difference at $p \leq 0.005$ from the saline vaccinated mice upon treatment with same concentration

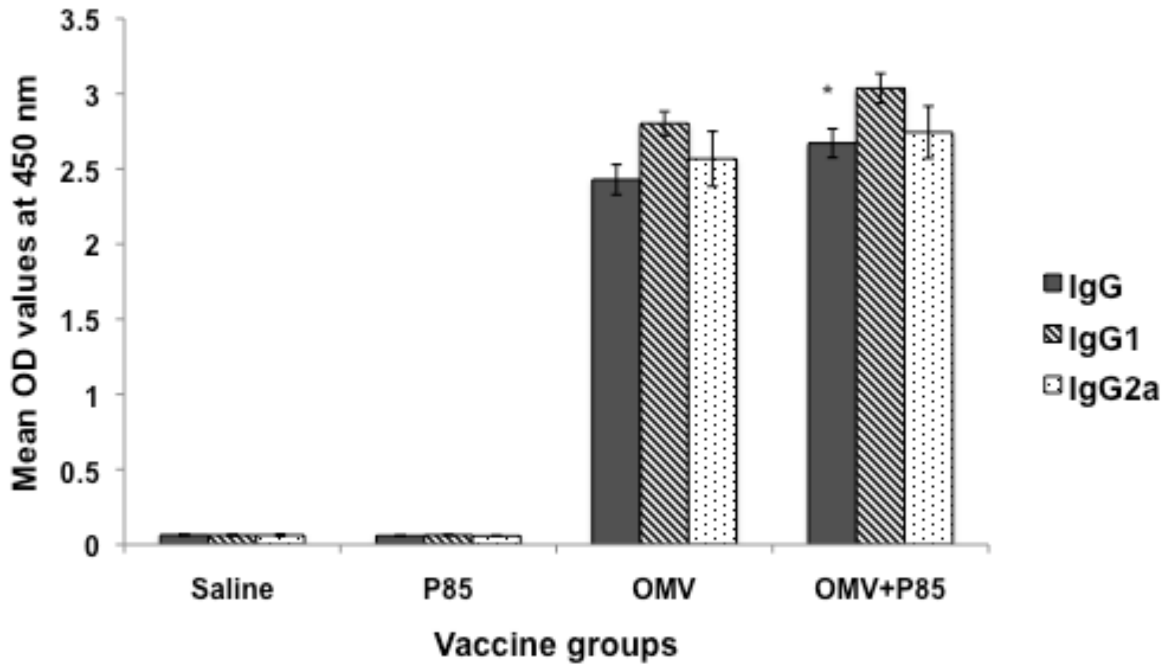

Figure 3.6: *B. melitensis* specific plasma antibody responses.

Antibody titers determined in the plasma of BALB/c mice vaccinated with OMVs or OMVs+P85. Levels of total IgG, IgG1 and IgG2a were determined using an indirect ELISA, using a 1:6400 dilution of plasma. Data represents results from 5 mice per group and each sample tested in duplicates. The graph represents the mean ± SEM of absorbance at 450 nm of the color developed. Asterisk (*) indicates the statistically significant difference ($p \leq 0.01$) between OMVs and OMVs + P85 vaccinated mice.

Table 3. 1: Protection provided by OMVs against *B. melitensis* challenge in BALB/c mice.

A Vaccine	Adjuvant	*Brucella* load in spleen Log$_{10}$ CFUs (mean ± SEM)
OMVs	None	4.23 ± 0.11
OMVs	IFA	4.09 ± 0.31
OMVs	0.003% P85	3.94 ± 0.21
OMVs	0.03% P85	3.47 ± 0.14**
OMVs	0.3% P85	3.97 ± 0.31

B Vaccine	Adjuvant	*Brucella* load in spleen Log$_{10}$ CFUs (mean ± SEM)
Saline	None	5.71 ± 0.11
OMVs	None	4.31 ± 0.08*
None	0.03% P85	4.75 ± 0.09*
OMVs	0.03% P85	3.45 ± 0.06**

Mice were vaccinated with OMVs or OMVs + adjuvant or adjuvant alone. Saline vaccinated mice group was the negative control. After challenging the vaccinated mice with *B. melitensis*, *Brucella* CFUs in spleens were determined as an indicator of the efficacy of vaccine. Top panel represents the potential of IFA and P85 as an adjuvant to enhance efficacy of OMVs as vaccine. Bottom panel presents the efficacy of OMVs+P85 as vaccine in mice in comparison to non-vaccinated mice against *B. melitensis* challenge. The data represents the mean ± SEM of CFUs of *Brucella* in spleen from 5 mice per group. One asterisk (*) represents statistically significant difference at $p \leq 0.01$ from saline vaccinated mice and two asterisks (**) represent

statistically significant difference at $p \leq 0.01$ from saline vaccinated mice as well as OMVs vaccinated mice.

Chapter 4

Adjunct immunotherapy against chronic *Brucella melitensis* infection in mice

Manuscript submitted to the Vaccine

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Abstract

Outer membrane vesicles (OMVs) from *Brucella melitensis* and irradiated *Brucella neotomae* have been shown to be effective vaccines against *B. melitensis* challenge in a mouse model. The present study shows the efficacy of these two vaccines as therapeutic vaccines in combination with conventional antibiotics against *B. melitensis*. Chronically *B. melitensis* infected female BALB/c mice were treated for 4 weeks with doxycycline and gentamicin and were vaccinated twice during the course of therapy. Although no additive effect of vaccine and antibiotics was seen on the clearance of *B. melitensis*, mice receiving vaccines along with antibiotics showed a control of *Brucella* replication post-treatment compared to the mice that were treated with only antibiotics. Administration of irradiated *B. neotomae* along with antibiotics lead to higher production of IFN-γ *ex vivo* by splenocytes upon stimulation with heat inactivated *B. melitensis* while no such effect was seen in mice vaccinated with OMVs. However, OMV vaccinated mice developed significantly higher IgG antibody titers at the end of the

treatment compared to the mice that received only antibiotics. The mice that received only vaccine did not show any clearance of *Brucella* from spleens and livers and thus the vaccines failed to act as therapeutic. This study suggests that incorporating OMVs or irradiated *B. neotomae* along with conventional antibiotics might be able to improve the therapeutic efficacy and control the progression of disease in treatment failure cases and further prevent relapses.

Introduction

Brucellosis is the most common bacterial zoonotic disease worldwide [1]. Treatment of animal brucellosis is not economically feasible and these infected animals and their products remain the source of infection for humans [2]. The World Health Organization's (WHO) recommendation for treatment in humans involves combination antibiotic therapy for 6-8 weeks [3]. Even after prolonged treatment, the rate of treatment failure is 1-5% and the relapse rate remains as high as 5-10% in humans [2]. Reasons for such high treatment failure are not completely understood. Patient non-compliance due to the long duration of therapy can be the major cause for treatment failure thus shorter therapy duration is desirable. In the case of relapses, isolated *Brucella* usually are sensitive to the same antibiotic treatment, thus showing absence of drug resistance [4]. Thus, the relapse occurs because of re-growth of *Brucella* as the immune system failed to control the growth of pathogen after treatment. There is a need for an alternative therapeutic option other than conventional antibiotics. This should help to reduce the duration of therapy and side effects, as well as prevent therapeutic failures and relapses. During chronic infection in humans the immune system shifts from Th1 to Th2

type and thus cannot eliminate infected cells or intracellular *Brucella* [5, 6]. In experimental mouse infection, it has been shown that *Brucella* infected BALB/c mice show severe disruption of spleen morphology and depletion in CD4+ and CD8+ lymphocytes which are required to eliminate the infected cells [7]. In both situations there is a clear case for immunotherapy in the treatment of chronic brucellosis that would stimulate the immune system to fight against *Brucella*. If the immune system can be stimulated and used in synergy with antibiotic treatment, it may be possible to reduce the duration of treatment of brucellosis. Moreover, if a stronger immunity can be generated using specific immuno-stimulants against *Brucella* during therapy, it may also prevent relapses after the end of treatment. The choice of such immuno-stimulant is critical in that it should not create non-specific immune stimulation and should be well tolerated by the infected host. It has been shown previously that DNA vaccines for *Mycobacterium tuberculosis* when used in combination with conventional chemotherapeutics switched the immune response in chronically infected mice from Th2 to Th1 and prevented relapse after treatment [8]. Use of immuno-stimulants, like levamisole and IFN-α 2a for the treatment of human brucellosis has been reported but with mixed results [9, 10]. Most of the approved vaccines to control *Brucella* infections are live, attenuated strains and cannot be used in infected hosts because of safety concerns. Moreover, it has been found that live-attenuated *B. abortus* strain RB51 given to *B. abortus*-infected mice cleared faster than from non-infected mice and failed to provide clearance of *B. abortus* (Contreras-Rojas A, Schurig G., unpublished data). There is a need for a non-infectious vaccine candidate that can provide a specific immuno-stimulation to treat *Brucella* infections. Therefore, we tested the potential of

outer membrane vesicles (OMVs) obtained from *B. melitensis* and irradiated *B. neotomae* as immuno-stimulants to treat *B. melitensis* infection in mice. OMVs are double membrane structures that are naturally released by Gram-negative bacteria [11]. OMVs are mainly comprised of the outer membrane, periplasmic proteins, LPS and inner membrane [12]. We have shown previously that OMVs provide protection against *B. melitensis* challenge in mice [13] that can be further enhanced by adding Pluronic P85 to OMVs (manuscript submitted).

B. neotomae was first isolated in 1957 from desert wood rats in the western United States [14]. So far its potential to cause disease in humans or other animal species is not known. Recently gamma irradiated *Brucella neotomae* (IBN) has been shown to protect against all pathogenic species of *Brucella* that cause infection in a mouse model [15]. Both these vaccine candidates are safer than live-attenuated vaccines that have the risk of reverting back to wild type. In the present work our goal was to test the efficacy of *Brucella* OMVs and irradiated *B. neotomae* as therapeutic vaccines to cure *B. melitensis* infection in mice.

Materials and Methods

<u>Bacterial strains:</u> Wild type *B. melitensis* 16M (our stock culture) was used to obtain OMVs and as well as to infect mice. *B. neotomae* strain 5K33 was purchased from American Type Culture Collection (ATCC).

<u>Preparation of vaccines:</u> OMVs were obtained from smooth strain *B. melitensis* 16M as previously described [13]. Briefly, stock cultures of *B. melitensis* 16M was streaked on

TSA plates and incubated for 48 hr. Using cotton swabs, bacterial culture was transferred from plates to TSA slants containing 0.7% yeast extract and allowed to grow for 48 hr. Next, the bacterial culture was scraped from slants using TSB and collected and spread on TSA plates (150 mm x 15 mm, 100 plates) containing 0.7% yeast extract. After an incubation of 48 hr, the bacterial culture was scraped using 200 µL PBS/plate. The culture was centrifuged at 15,191x g for 30 min and supernatant was collected. The supernatant was filter sterilized twice using 0.22 µm filters, and the sterility of the filtrate was tested by inoculating 50 µl in 10 ml TSB and incubating at 37°C for 48-72 hr. Absence of any bacterial growth confirmed the sterility of the filtrate. To obtain the OMVs, the filtrate was ultracentrifuge at 176,508x g for 2 hr at 4°C in a Optima L-90K Ultracentrifuge (Beckman Coulter, Brea, CA). The pellet was washed in 50 mL PBS and centrifuged as originally. The washing step was repeated twice and finally the pellet was resuspended in 250 µL PBS and stored at -20°C. Concentration of protein in OMVs was determined using Pierce BCA protein Assay kit (Thermo Scientific) according to the manufacturer's protocol. For examining by the electron microscope, OMVs were mixed with 2% aqueous uranyl acetate solution for negative staining and placed onto 200-mesh formvar, carbon coated copper grids (Electron Microscopy Sciences). Excess liquid was soaked away using filter paper and the samples were viewed at 63,000x or 100,000x magnification on a Zeiss 10CA Transmission Electron Microscope (Virginia-Maryland Regional College of Veterinary Medicine). To determine the protein profile of OMVs, first OMVs were mixed with different concentrations of P85 (dissolved in phosphate buffered saline) using a double hub-syringe and then mixed with 2X Laemmli buffer (Bio-Rad) containing β-mercaptoethanol and heated for 5 min at

95°C. The samples were electrophoresed on 10% SDS-PAGE gels (Invitrogen) and stained with Coomassie blue to visualize protein bands.

B. neotomae was grown in TSB to mid log phase and spun down, aliquots containing 5 x 10^{11} CFUs/mL were stored at -80°C until use. Two to three weeks before immunization, aliquots of the vaccines were exposed to 350 krads of gamma irradiation using a ^{60}Co source irradiator (Gammacell 220 Irradiator). The inability of the irradiated bacteria to replicate was confirmed by plating on TSA and incubating for at least 7 days. The irradiated bacteria were stored at 4°C until used for immunization. Metabolic activity of irradiated *B. neotomae* (IBN) was accessed using Alamar blue as previously described [15]. Briefly, irradiated samples were washed in PBS twice and resuspended in TSB and mixed with Alamar blue at a ratio of 10:1 and incubated at 37 °C for 1 hr, a change in color from blue to pink was monitored.

<u>Mice infection and treatment:</u> Approximately 6-8 weeks old female BALB/c mice were infected i.p. with *B. melitensis* 16M (5 x 10^4 CFUs/mouse). After 7 weeks post-infection, mice were treated according to the scheme shown in Figure 4.3. Doxycycline and gentamicin were given at the dose of 3 mg/kg body weight and 5 mg/kg body weight respectively once daily injected i.p. A total volume of 100 μl containing 5 μg (protein) OMVs, 0.03% P85 and PBS was injected i.m. Approximately, 5 x 10^8 CFUs equivalent of irradiated *B. neotomae*/mouse was injected i.p. Negative control groups of mice were treated with saline administered i.p. Groups of mice were euthanized either after 14 days of treatment, at the end of the treatment or 4 weeks after the end of the treatment. Spleens and livers were collected and homogenized and serial dilutions were prepared

in TSB. The dilutions were spread on TSA plates and *Brucella* CFUs were determined after incubating the plates at 37°C for 3-4 days.

Cytokine levels in plasma: Mice were bled retro-orbitally, every week before the start of treatment and then during the 1st and 3rd week of treatment and finally after 1st and 3rd week post treatment (5th and 7th week from the start of treatment); blood was collected in heparanized tubes. Plasma were collected after centrifuging the blood samples at 13,000 g for 10 minutes and immediately stored at -80°C. To determine the levels of IFN-γ, Mouse IFN-γ (AN-18) ELISA Set (BD Pharmigen) was used according to the manufacturer's instructions.

Antibody titers: An indirect ELISA was performed to measure the levels of *B. melitensis* specific antibodies in the plasma of the mice. Briefly, heat inactivated *B. melitensis* 16M (stock culture was heated at 80°C for 1 hr, inactivation was confirmed by sterility of the culture after 48 hr) at the protein concentration of 1.0 µg/mL or 1 in 10 purified *Brucella* LPS (2 mg/mL) were adsorbed to wells of polystyrene plates (Nunc Maxisorp) in 50ul of bicarbonate buffer (pH 9.6). After incubating overnight at 4°C, plates were washed 4 times with phosphate buffer saline (PBS) containing 0.05% Tween-20. Then the wells were blocked with 2% bovine serum albumin in PBS and incubated for 2 hr at room temperature; the plates were washed 4 times as before. For the pre-treatment samples, plasma samples were diluted at 1:100 and added in duplicate to the wells and incubated for 3 hr at room temperature. To measure the specific titers a series of plasma dilutions were prepared and tested. Again, the plates were washed 4 times and isotype specific

goat anti-mouse horseradish peroxidase conjugates added for 30 min at room temperature. After washing the plates 4 times, 100 μL of TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated in dark for 20 min. The reaction was stopped by adding 100 μL/well of 0.18 M sulfuric acid, and the absorbance of the developed color was measured at 450 nm. The end point titers were obtained as the reciprocal of the highest dilution that showed a higher absorbance than absorbance of the pre-infected plasma samples.

Splenocyte culture and cytokine determination: At the end of the treatment splenocytes from treated mice (3 mice per group) were obtained as previously described [16]. Approximately, 5×10^5 splenocytes/well were seeded in flat bottom 96-well plates and stimulated with heat inactivated *B. melitensis* 16M (MOI of 1:1, 1:10 and 1:100). Splenocytes were stimulated with medium alone as negative control while with Concavalin A (1μg/mL) as positive control. After incubating cells for 5 days at 37°C, supernatants were collected and the concentration of IFN-γ was determined using cytokine ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

Statistical analysis: All data were analyzed using Microsoft Excel and student's t test was used to determine *p* values.

Result

Characterization of vaccine candidates: Electron microscopy revealed the characteristic double membrane structure of OMVs (data not shown). A Commassie blue-stained SDS-PAGE gel showed the same number and sizes of proteins as shown previously [13]. Gamma irradiated *B. neotomae* (IBN) when cultured in TSB showed the absence of viability while Alamar blue test showed the presence of metabolically active cells.

Mouse model: To the authors' best knowledge, there is no established mouse model for the immunotherapy studies and relapse related to *Brucella* treatment. We purposely treated mice with lowest recommended therapeutic dose of doxycycline (3 mg/kg body weight) so that mice treated only with antibiotics resulted in therapeutic failure. This would help in measuring any additive effects vaccines have along with antibiotics in clearing *Brucella*. In addition it would help in further studying the effect of vaccination during treatment on the replication of *Brucella* post-treatment. Mice were treated after 7 weeks of infection because they were then considered to be in the chronic phase of *Brucella* infection, as there is a plateau in *Brucella* CFUs in organs such as the spleen and liver [17]. Mice were given vaccinations twice, two weeks apart, during the course of therapy (Figure 4.3). Mice were euthanized at three time points, before the boost vaccination, at the end of therapy and 4 weeks after the end of therapy. This should help in determining the additive effect of antibiotics and vaccine and post therapy effects on chronic infection. Also, at the end of treatment splenocytes from treated mice were stimulated with heat inactivated *B. melitensis* 16M to determine splenocyte proliferation

and cytokine production as an indication of the immune status of the mice. Antibody titers were determined in the plasma to determine the effect of vaccinations.

Vaccine associated sickness: *B. melitensis* infected mice that were given OMVs mixed with P85 as an adjuvant exhibited vaccine-related sickness. However, the mice recovered in 3 days and there was no significant weight loss indicative of illness. This effect might be due to *Brucella* LPS. Mice that were given IBN did not show any sign of sickness throughout the study.

Levels of IFN-γ in plasma: Levels of circulating IFN-γ increased within the first week of infection (Figure 4.1). There was a sudden drop at week two and then an increase at the 3rd week post-infection. In untreated mice, these levels remained constant until the 8th week and then dropped at the 10th week. In case of treated mice the IFN-γ levels in mice plasma after the 1st, 3rd, 5th and 7th weeks from the start of the treatment followed the trends of levels of *Brucella* infection in mice. Thus it appeared that the higher the *Brucella* CFUs in the organs, the higher the levels of IFN-γ in plasma. Following the 1st, 3rd and 5th week from the start of treatment all the mice that received antibiotics showed significantly lower levels of circulating IFN-γ. But following the 7th week only the mice treated with antibiotics + vaccines had lower circulating IFN-γ.

Mouse treatment: Table 4.1 shows *Brucella* CFUs in the spleens and livers of mice after receiving different treatments. At 2nd and 4th weeks after treatment there is a significant decrease in *Brucella* CFUs per organ in mice treated with doxycycline and gentamicin

compared to saline treated mice. But there is no additive effect when any vaccine was given along with chemotherapeutics. Moreover, there is no reduction in *Brucella* CFUs in the mice that received only vaccine. At four weeks post-treatment, compared to *Brucella* CFUs at the end of treatment, there is an increase both in spleen and liver CFUs in mice treated only with antibiotics while the CFUs are unchanged in the mice receiving vaccine along with antibiotics. Not surprisingly, the *Brucella* CFUs decreased with time in non-treated (saline treated) mice, as after 12-14 weeks of infection, there is a declining phase of chronic *Brucella* infection in mice [17].

<u>Antibody titers in plasma:</u> Antibody titers increased significantly from the 1st week to 2nd week post-infection but then persisted until the end of the study in non-treated mice (Figure 4.2). Treatment with antibiotics significantly increased the IgG titer in treated mice (Figure 4.6A). Compared to the IgG titers in the mice treated with only antibiotics, the higher titers were measured in mice treated with antibiotics plus OMVs. However, administration of irradiated *B. neotomae* did not raise the antibody titers specific to *B. melitensis*. Mice vaccinated with OMVs also showed higher titers of IgG to purified *Brucella* LPS compared to DG treated mice.

<u>Levels of IFN-γ in splenoctyes culture supernatants:</u> Splenocytes from all mice expressed IFN-γ upon stimulation with heat inactivated *B. melitensis*. Levels of IFN-γ produced were increased as a function of the MOI of heat inactivated *B. melitensis* 16M as treatment in all the groups showed a dose dependent activation of splenocytes. Instead of comparing treated mice with saline treated mice, mice treated with DG and

DG and vaccines were compared. All the mice that received antibiotics with or without vaccines, showed similar intracellular *Brucella* CFUs at the end of the treatment. Thus the effect of intracellular *Brucella* loads on the activation and cytokine production could be discounted as a responsible factor. Levels of IFN-γ in splenocyte cultures were higher in mice treated with DG + IBN compared to mice treated with DG alone (Figure 4.5). While splenocytes from mice treated with DG and vaccinated OMVs showed lower levels of IFN-γ upon stimulation with heat inactivated *B. melitensis*.

Discussion

Eradication programs have resulted in successful control of brucellosis in developed countries but the disease is still a burden in the developing world [18]. Although the disease in humans is rarely fatal, it can be severely debilitating and disabling [19]. The WHO recommendation for human treatment has not been updated in the last 25 years. Treatment with a combination of chemotherapeutics for 6-8 weeks is recommended. However, this regimen is still associated with high treatment failure and relapses [4]. In the case of humans, treatment failure is considered as the persistence of signs and symptoms at the end of scheduled therapy. Relapse is defined as the reappearance of signs and symptoms or positive blood cultures within one year of treatment. In the case of experimental murine brucellosis, treatment failure is characterized by the presence of *Brucella* in targeted organs like spleen, liver and lung at the end of the therapy. There is no relapse model following treatment of *Brucella* infection in mice.

Among several other factors that might lead to relapse in humans is the start of the treatment within 10 days from the time of *Brucella* infection. Decreased antigenic

stimulus due to early treatment leads to relapses [4]. Thus, it is important that the host develops immune responses against *Brucella* to make therapy more successful and to prevent relapses. Also in experimental brucellosis it has been shown that if antibiotic treatment is started immediately after inoculation of *Brucella* it causes reduced clearance of *Brucella* in mice [20]. This is consistent with the development of protective immune responses along with the antibiotics as a requirement to eliminate *Brucella* from the host. *Ex vivo* cultures of T-lymphocytes from chronic brucellosis patients show T-cell anergy on stimulation with phytohaemagglutinin (PHA), *E. coli* LPS and heat killed *B. abortus* [21]. This T-cell anergy leads to the Th1 to Th2 switch in the immune response that fails to eliminate *Brucella* infection in humans [6]. Considering these factors, immunotherapy to boost the immune system in *Brucella* infected hosts could be a valuable adjunct to antibacterial chemotherapy. The concept of immunotherapy to treat *Brucella* infection in mice has been tested, but the results vary from one study to another. Levamisole, which is an antiparasitic drug, was widely tested in humans. Most published work on the use of levamisole for the treatment of brucellosis is in humans where comparisons were based on laboratory and clinical parameters and it was not possible to determine the clearance of *Brucella* from infected organs [22]. In an experiment where *B. melitensis* infected mice were given levamisole, no difference was found in the clearance of *Brucella* from the spleens [23]. Use of exogenous interferon has been also reported to be useful for the treatment of brucellosis in humans but the reports are not compelling. Use of both levamisole and interferon as an immunostimulator for the treatment of brucellosis is limited because of the non-specific stimulation caused by these compounds. In the present work we aimed to test the

Brucella vaccines that will induce specific stimulation and could be used for the treatment of brucellosis. Outer membrane vesicles (OMVs) obtained from *B. melitensis* could be good candidate to be used as a therapeutic vaccine as it eliminates the risk associated with live-attenuated strains. Gamma irradiated *B. neotomae* could be another option, as it is not known to be pathogenic in humans and after irradiation the strain loses the ability to replicate.

Previously it has been shown that both OMVs and irradiated *B. neotomae* provided protection in mouse model against challenge with *B. melitensis* [13, 15]. In both cases, a stronger Th1 response than Th2 response was found to be involved. Higher levels of IFN-γ compared to IL-4 were detected in splenocyte culture supernatants from mice vaccinated with either OMVs or irradiated *B. neotomae*. Furthermore, we showed that pluronic P85 enhanced the efficacy of OMVs as preventive vaccine against *B. melitensis* (manuscript submitted). This enhancement was the result of non-specific immune stimulation generated by P85 and the higher antibody titers induced by OMVs+P85.

Typically, *Brucella* infection in mice has been divided into three major phases: first is the incubation phase which lasts for 2-3 days after initial infection (reviewed in [17]). Next is the acute phase for 2-3 weeks, when *Brucella* actively replicates in different organs of the reticuloendothelial system. Last is the chronic phase of infection that is again divided into plateau phase (6-8 weeks) where *Brucella* CFUs in organs reaches a plateau phase and late chronic phase characterized by a decline in *Brucella* CFUs that can persist up to 6 months to 12 months. Circulating IFN-γ in the blood of infected mice correlates with these phases. Thus, the higher the CFUs of *Brucella* in spleens, higher

the levels of IFN-γ in plasma are. Our data showing levels of circulating IFN-γ follows the previously reported trend of *Brucella* loads in spleens [24, 25]. There is a sudden increase in levels of IFN-γ following the 1st week of the infection. Also a significant increase from the 2nd to 3rd week agrees with the IFN-γ levels shown before in *ex vivo* splenocytes cultures from the infected mice stimulated with *Brucella* antigens [24]. But in *ex vivo* experiments a gradual increase in the levels of IFN-γ occurred during the 3rd to 8th week post infection with the highest peak at 8th week; however, in this study we found IFN-γ levels to be constant from the 3rd to 8th week. Higher levels of IFN-γ were detected in plasma almost until the end of plateau phase (i.e. 8 weeks post infection when *Brucella* CFUs remained almost constant in spleens), followed by a decline (Figure 4.1). Similar to *ex vivo* experiments, there is a sharp decline in IFN-γ levels at the 10th week post infection, which is also almost the end of plateu phase and the start of the declining phase of *Brucella* CFUs.

We chose the time between the 7-8th week to start treatment as the infection had not yet reached the declining phase, and the impact of therapeutics on mice treatment could be well differentiated from non-treated mice. Mice were treated with antibiotics; doxycycline (3 mg/kg body wt.) and gentamicin (5 mg/kg body wt.), for 4 weeks and vaccines were given twice during therapy. We have previously shown that DG combination clears *Brucella* from infected mice more efficiently than doxycycline alone (manuscript submitted). We used the lowest recommended dose of doxycycline so that any additive effect of the administered vaccine could be determined. Moreover a therapeutic failure would likely result at the end of the treatment and allow us to study the effect of vaccines on subsequent replication of remaining *Brucella* after the end of the treatment.

The mice were treated for a month to allow them to develop immune responses to the primary and booster dose of vaccines.

There was no additive effect of chemotherapeutics and vaccines on the clearance of *Brucella* from infected mice after treatment for 2 or 4 weeks (Table 4.1). Vaccines when given alone failed to clear any *Brucella* from the mice. This shows that the tested vaccines cannot be used solely as therapeutic vaccines. No significant differences in the clearance of *Brucella* were seen between chemotherapeutics vs. chemotherapeutic plus vaccines at the end of the treatment. However, significant differences were obtained 4 weeks after the end of the treatment. There was no increase in *Brucella* CFUs after the end of treatment in the mice that were given both antibiotics and vaccines. Moreover, a major increase in *Brucella* CFUs was seen in the mice that were given only antibiotics 4 weeks after end of treatment. This strategy of treatment using vaccines and antibiotics might be helpful to control the multiplication of *Brucella* after therapeutic failures and could help prevent relapses.

Figure 4.4 shows the levels of IFN-γ in plasma at different time points during and after therapy. The levels are directly proportional to the *Brucella* CFUs in spleens. Thus, the higher the *Brucella* CFUs in spleens, the greater the levels of circulating IFN-γ are. The levels of circulating IFN-γ decreased within one week of treatment in mice treated with antibiotics compared to untreated mice or mice treated with only vaccines. Although these lower levels of IFN-γ were maintained in all the groups that received antibiotics. At 7th week (3 weeks after the end of treatment) IFN-γ levels went up in mice treated only with antibiotics. This indicates the replication of remaining *Brucella* occurred after the

end of the therapy. In mice treated with both antibiotics and vaccines, the IFN-γ levels remained significantly lower than untreated mice indicating lower replication of *Brucella.*

To determine the effect of administration of vaccines to *Brucella*-infected mice on the immune status of mice, splenocytes from mice were stimulated with *Brucella* antigens and levels of IFN-γ production were measured. Mice vaccinated with IBN produced more IFN-γ upon stimulation with heat inactivated *B. melitensis* 16M (Figure 4.5). This explains the ability of mice treated with antibiotics + IBN to prevent the multiplication of *Brucella* after the end of treatment. While in the case of OMV-vaccinated mice, splenocytes produced less IFN-γ upon stimulation with OMVs or heat inactivated *B. melitensis*. Mice that received only IBN showed higher induction of splenocytes and production of IFN-γ (data not shown) but failed to clear the *Brucella*. Thus, stimulation of the immune system, at least to the degree IBN or OMVs in the present study could do, is not enough to clear a *Brucella* infection in mice.

After treatment with antibiotics the mice showed higher titers of plasma IgG compared to untreated mice (Figure 4.6A). This finding agrees with that previously reported for *B. melitensis* infected mice treated with doxycycline and streptomycin as a significant increase in antibody titers after antibiotic treatment was observed [26]. Also, human brucellosis patients show higher or persistent levels of antibodies after successful treatment [27, 28]. This might be due to the intracellular killing of *Brucella* by antibiotics in the antigen presenting cells and presentation of antigens that lead to humoral responses. IgG antibody titers against *B. melitensis* increased significantly in the mice treated with DG + OMVs. OMVs contain a large amount of LPS and antibodies specific to *Brucella* LPS were detected in high concentration in the mice vaccinated with OMVs

(Figure 4.6B). It has been shown that antibodies to the O-side chain in LPS can provide protection against *Brucella* challenge after passive transfer in mice [29-31]. This explains the control on *Brucella* replication after the end of treatment in mice treated with DG + OMVs. Mice treated with antibiotics + IBN showed lesser levels of IgG compared to DG treated mice; this might be due to the negative regulation of Th1 type immune responses during the induction of humoral immunity. Administration of levamisole or interferon-alpha 2b along with chemotherapeutics also resulted in decreased titers of antibody in the treated human patients compared to those that were treated only with antibiotics [10].

In conclusion, IBN and OMV vaccines given along with antibiotics enhanced different mechanisms of immune responses but both provided significant control in the growth of *Brucella* after the end of therapy. Mice not treated with antibiotics, but only receiving IBN or OMVs showed higher IFN-γ levels and IgG titers respectively, failed to clear the infection. Thus, initial chemotherapy is required and use of these vaccine candidates alone for therapy against *Brucella* infection is not suitable. Future efforts should be directed in developing a laboratory animal model to study relapse after treatment of brucellosis and to further test the efficacies of therapeutics to prevent relapses.

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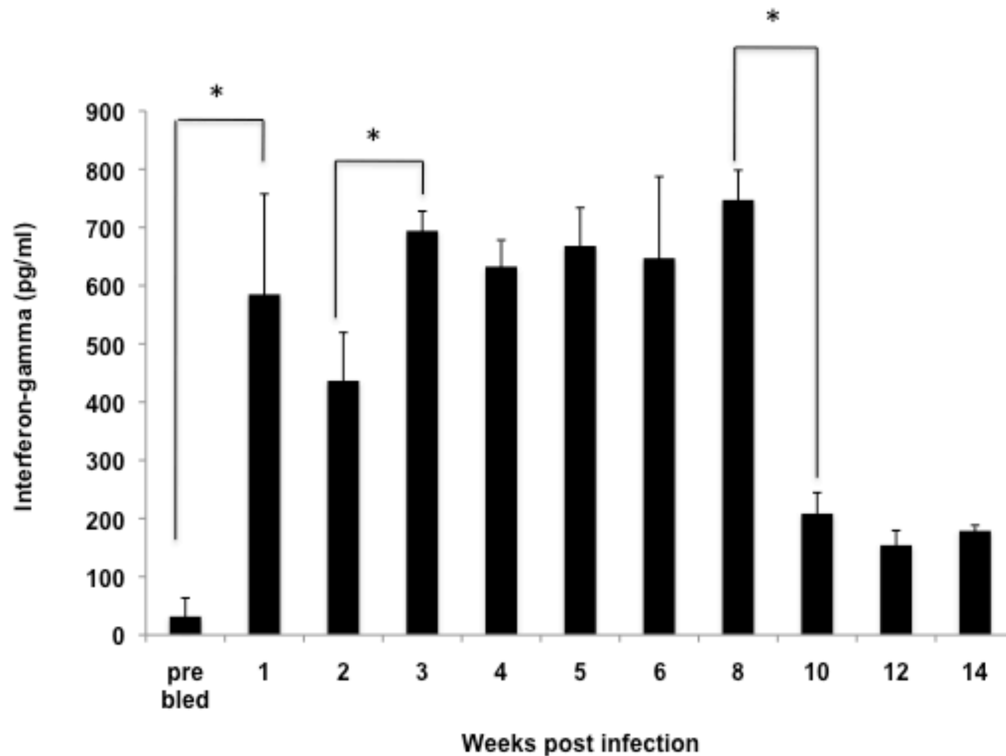

Figure 4.1: Levels of IFN-γ in plasma of *Brucella melitensis* infected mice.

Mice (n=5) were bled retro-orbitally and plasma was collected immediately. Concentration of IFN-γ was measured using indirect sandwich ELISA. Plasma samples were diluted 1:10 in assay diluent, and the range of detection was between 200 pg/mL to 3.1 pg/mL. Data represent the mean± SEM and asterisk (*) represents statistically significant difference at $p \leq 0.005$ between the groups compared.

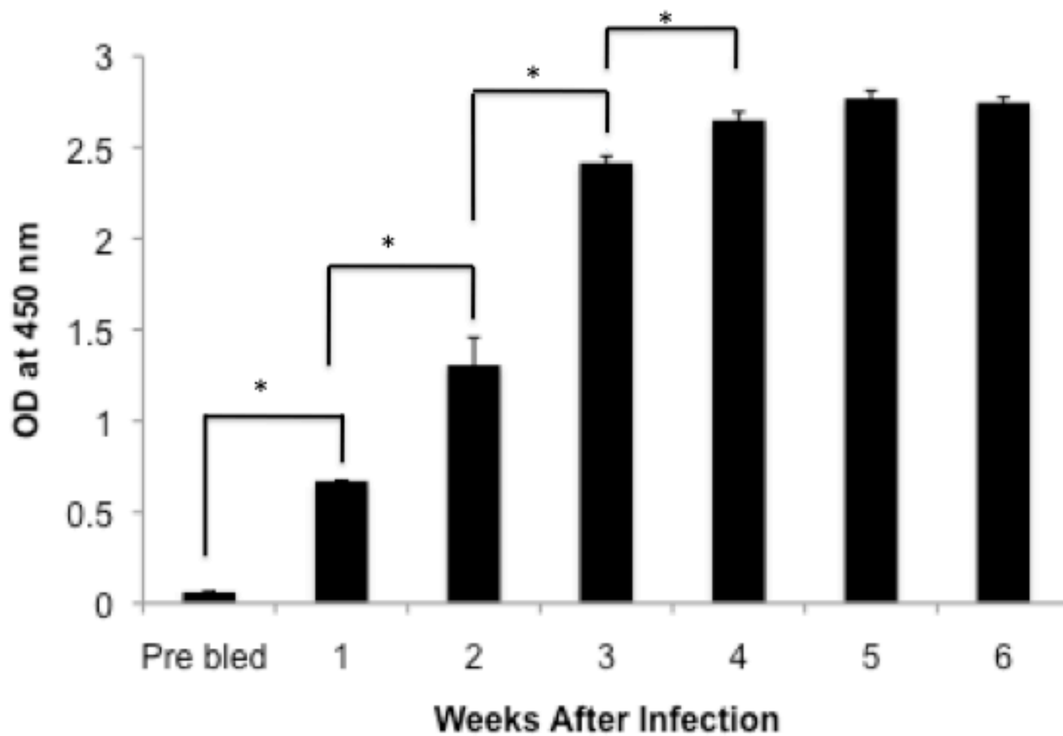

Figure 4.2: Levels of IgG antibody in plasma of *B. melitensis* infected mice. Mice were bled retro-orbitally and plasma was collected every week. A dilution of 1:200 of plasma samples was prepared and assayed for the presence of IgG antibodies using an indirect ELISA. The data represents the mean ± SEM (n=5) of absorbance at 450 nm of the color developed. Asterisk (*) represents the statistically significant difference ($p \leq 0.005$) between the compared groups.

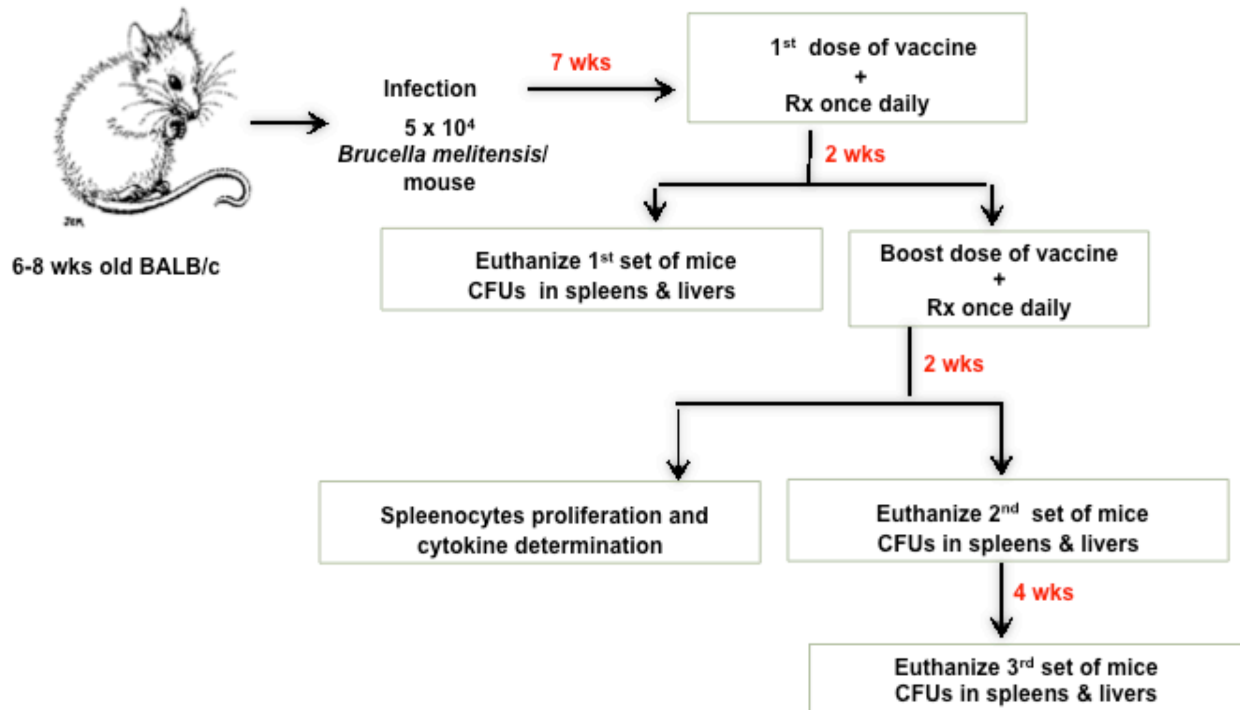

Figure 4.3: Mice infection, sampling and treatment scheme.

After 7 weeks of *Brucella* infection, mice were treated with doxycycline (3 mg/kg body wt.) and gentamicin (5 mg/kg body wt.) for 4 weeks. Mice treated with saline were the negative controls. During the course of chemotherapy designated groups of mice were given vaccine either OMVs + P85 or irradiated *B. neotomae* at 1st $+14^{th}$ day of the treatment. Two groups of mice were given vaccine only while one groups received only chemotherapeutic.

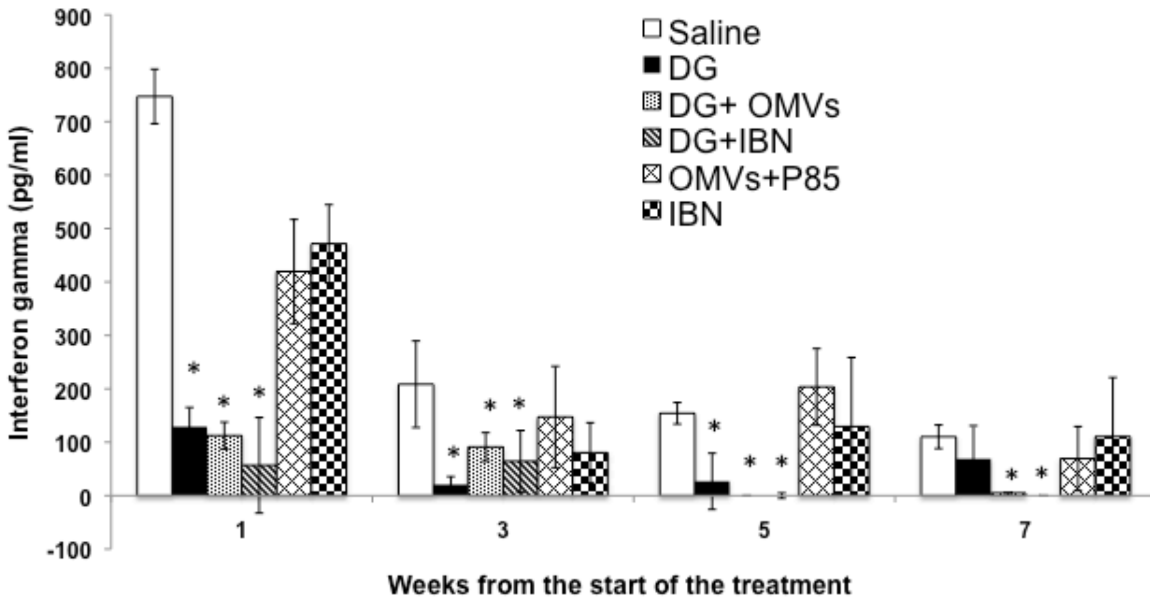

Figure 4.4: Effect of treatments on levels of circulating IFN-γ in mice.

Mice were bled retro-orbitally 1st, 3rd, 5th and 7th week from the start of the treatment and levels of circulating IFN-γ were determined in each group (n=4) by indirect sandwich ELISA. All samples were tested in duplicates. Limit of detection was 3.1 pg/mL. The data represents mean ± SEM and asterisk (*) represents the statistically significant differences ($p \leq 0.01$) from the saline treated mice during the same week.

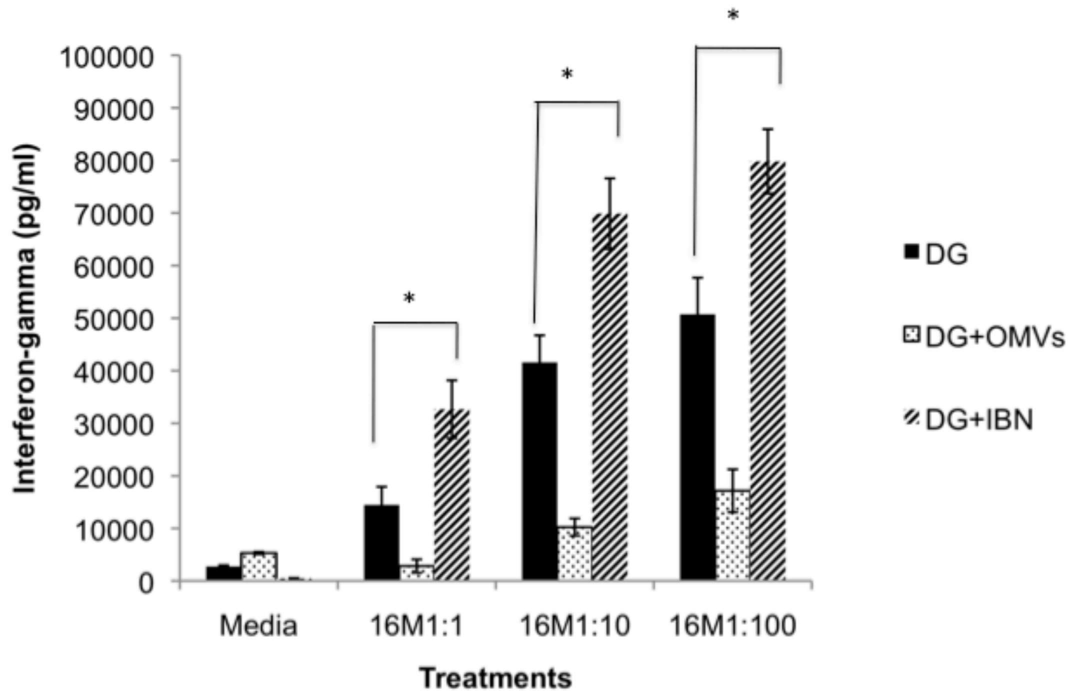

Figure 4.5: Levels of IFN-γ produced by splenocyte from treated mice after stimulation with heat inactivated *B. melitensis*.

Splenocytes from doxycycline-gentamicin (DG), DG + OMVs and DG + IBN treated mice (n=3), were stimulated with heat inactivated *B. melitensis* 16M (MOI 1:1, 1:10 and 1:100) for 5 days. Levels of IFN-γ in the culture supernatants (1:100 dilution) were measured using indirect sandwich ELISA. All the samples were tested in triplicate. The data represents the mean ± SEM and asterisk (*) represents statistically significant difference ($p \leq 0.001$) between the groups compared.

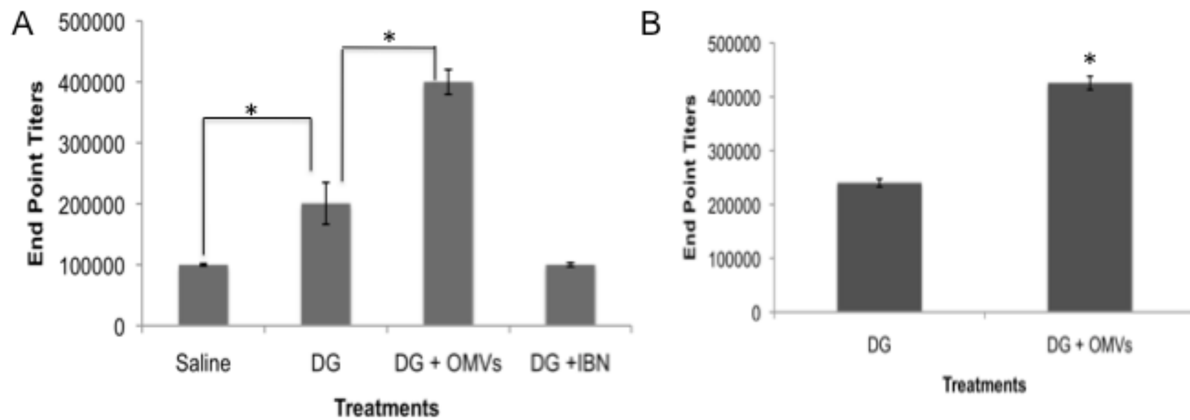

Figure 4.6: Titers of total IgG antibody in plasma of mice at the end of the treatment.

Mice (n=5) were bled retro-orbitally and plasma was collected. For determining the antibody titers, equal volume of plasma was pooled together from the mice in the same group and tested in triplicates. An indirect ELISA was performed and the antibody titers were determined as the reciprocal of the highest dilution of the pooled sample that yielded absorbance higher than the non-infected mice sample. A: antibodies to heat inactivated *B. melitensis* 16M and B: antibodies to purified *Brucella* LPS. Asterisk (*) represents the statistically significant difference ($p \leq 0.01$) between the groups compared.

Table 4. 1: *Brucella* CFUs in spleens and livers of the treated mice at different time points.

A Treatment/CFUs in spleens	2 weeks after treatment	4 weeks after treatment	4 weeks after the end of treatment
Saline (Control)	5.8 ± 0.57	4.8 ± 0.21	3.91 ± 0.42
Doxycycline-Gentamicin (Dox-Gen)	4.1 ± 0.22*	2.9 ± 0.15*	5.18 ± 1.00
Dox-Gen + OMVs	3.7 ± 0.14*	2.8 ± 0.06*	2.81 ± 0.63**
Dox-Gen + Irradiated *B. neotomae*	3.8 ± 0.24*	2.5 ± 0.28*	2.09 ± 0.43**
OMVs	5.1 ± 0.73	4.9 ± 0.19	4.31 ± 0.2
Irradiated *B. neotomae*	5.8 ± 0.55	4.9 ± 0.6	5.42 ± 0.94

B Treatments / CFUs in livers	2 weeks after treatment	4 weeks after treatment	4 weeks after the end of treatment
Saline (Control)	4.4 ± 0.41	4.1 ± 0.53	2.29 ± 0.49
Doxycycline-Gentamicin (Dox-Gen)	2.3 ± 0.81*	1.8 ± 0.34*	3.26 ± 1.17
Dox-Gen + OMVs	1.8 ± 0.17*	1.6 ± 0.0*	1.84 ± 0.27**
Dox-Gen + Irradiated *B. neotomae*	2.1 ± 0.65*	1.6 ± 0.0*	1.60 ± 0.00**
OMVs	3.9 ± 0.17	3.5 ± 0.16	3.12 ± 0.49
Irradiated *B. neotomae*	4.1 ± 0.63	3.6 ± 0.44	4.3 ± 0.63

Mice were treated with doxycycline-gentamicin (DG), DG + vaccines or vaccines alone. Saline treated mice were the negative controls. Mice were euthanized after 2 and 4 weeks of treatment and 4 weeks after the end of treatment. *Brucella* CFUs were determined in spleens and livers by plating homogenized organs on TSA. The data represents mean ± SEM (n=5). Single asterisk (*) represents statistically significant difference ($p \leq 0.01$) from controls (saline) and double asterisk (**) represents statistically significant difference ($p \leq 0.01$) from both controls as well as from doxycycline + gentamicin (DG) treated groups.

Chapter 5

General Conclusions and Future Studies

The research presented in this dissertation focused on finding an effective treatment for chronic brucellosis in a mouse model. Brucellosis in humans consists of two phases: acute and chronic. The acute phase is characterized by undulant fever and asthenia while localized infections like endocarditis, spondylitis, encephalitis and erratic recurrent fevers are the characteristics of chronic infection (10). Despite early diagnosis and treatment, 10-30% of patients develop chronic brucellosis, which is difficult to treat (27). For reasons that are not fully understood, treatment failure and relapses associated with brucellosis remain as high as 1-5% and 5-10% respectively (30). Two factors that have been identified as reasons for brucellosis treatment failure and relapse are the lengthy duration of treatment (and associated patient non-compliance) and poor immune responses against *Brucella* (1). We focused on two major approaches to develop an effective treatment against *Brucella* infection in a mouse model. The first goal was to deliver a drug more efficiently inside phagocytic cells where *Brucella* is replicating. This should result in faster clearance of the pathogen and thus reduce the duration of treatment. The second goal was to incorporate immunostimulants with antibiotic treatment to treat *B. melitensis* infection in mice. This protocol should help to stimulate the immune system against *Brucella*, which in conjunction with antibiotics, would help in clearing *Brucella* and prevent the re-appearance of *Brucella* after the end of the treatment.

Antibiotics like gentamicin that poorly cross cell membrane barriers remain ineffective against intracellular pathogens like *Brucella* (7, 24). Different types of nanoparticles have been shown to cross cell membrane and deliver drugs or proteins or genes inside the cells (5, 26). We first tested the efficacy of gentamicin loaded block ionomer complexes (BICs) (Chapter 2.1). The complexes did not cause toxicity either *in vitro* or *in vivo*. The complexes showed high efficacy against *B. melitensis in vitro* but failed to eliminate *Brucella* from infected mice. We showed that a doxycycline-gentamicin combination is more efficacious in mice than doxycycline alone. But the doxycycline-BICs combination showed the same effect on *Brucella* clearance as a free gentamicin-doxycycline combination. We speculated that this inefficacy might be due to the burst release of gentamicin from the BICs after dissolution (Manuscript PAT-11-150.R2 accepted, Polymers for Advanced Technologies). Because of this burst release, most of the drug was released within first few hours of dissolution and an adequate amount of gentamicin-containing BICs did not reach the affected organs (e.g. spleens and livers). Assuming most of the drug was released from BICs and reached the targeted organs, the released drug should have acted at least as well as free gentamicin. However, mice treated with BICs-doxycycline combination showed significantly less clearance of *Brucella* from spleens and livers compared to mice treated with the free gentamicin-doxycycline combination. This shows that other factors regarding BICs are involved. The biodistribution and *in vivo* fate of BICs is an important avenue for future research.

Considering the limitations of BICs, the next goal was to create drug-loaded nanoparticles that would hold the drug more tightly and have a longer circulation time. Magnetite block ionomer complexes (MBICs) were created with block polymers adsorbed on nanomagnetites and then gentamicin was electrostatically attached. MBICs overcame the presumed early burst release and showed a constant release of drug for 40-60 hrs (Pothayee N. et. al., manuscript submitted). MBICs did not show any *in vitro* toxicity and showed high efficacy of killing intracellular *Brucella in vitro*. When used to treat mice in combination with doxycycline, the efficacies to clear *B. melitensis* from spleens and livers were not better than the free gentamicin-doxycycline combination (chapter 2.2). However, doxycycline-MBICs combination provided significantly better clearance from mice compared to doxycycline alone. MBICs showed the intensity average diameter as 64 nm. The recommended range of particles for prolonged circulation time is between 70-150 nm. We speculated that the smaller size of the MBICs reduced the circulation time in mice and could not clear the *Brucella* from spleens and livers effectively. Later, gentamicin loaded clusters of MBICs called "MBIClusters were generated. Compared to MBICs, MBIClusters were larger in size (intensity average diameter 109nm). MBIClusters again did not show any better efficacy than free gentamicin when used in combination with doxycycline to treat *B. melitensis* infection in mice (Chapter 2.3). Histopathological examination of kidneys obtained from mice treated with MBICs and MBIClusters showed the deposition of pigmented macrophages on the peri-renal adipose tissue. By Perl's Persian Blue staining, the pigment was found to be iron and was absent in the

mice treated with free gentamicin. This shows that the intracellular iron in the pigmented macrophages was from the MBICs that contain magnetite core.

Thus, we were able to show that different types of gentamicin loaded nanoparticles, BICs and MBICs were able to clear *Brucella in vitro* but did not show improved efficacy in mice compared to free antibiotic. One of the major reasons is the localization of the pathogen in the specific organs like the spleen and liver rather than in the systemic phagocytes. Phagocytes rapidly take up nanoparticles but these are circulating macrophages, not macrophages or other cell types are harboring *Brucella*. *Brucella* replicates in organs and inside specific cells; the nanoparticles are unable to reach these targets. Organ specific targeting should be the focus for future research to treat *Brucella* infections. One way to achieve this may be to incorporate targeting molecules like antibodies or heme onto the surface of nanoparticles. Antibodies specific to cell receptors could be useful but for that an understanding of the difference of cell surface receptors on *Brucella* infected cells and non-infected cells is necessary. Heme, an iron containing porphyrin, has been shown before to be useful for nucleic acid targeting to the liver (32). Free heme in the mammalian body concentrates in the liver following an intravenous injection (18) or binds to heme-binding proteins like hemopexin and albumin and is transported to liver (29). Thus, heme molecules attached to the surface of drug-loaded nanoparticles may trigger the migration of the whole complex to the liver.

It has been well documented that antigen-antibody complexes are transported to the organs of the reticuloendothelial system (liver, spleen and lungs) for

downstream processing and removal from the body (9, 12, 25). Thus, coating nanoparticles with *Brucella* antigen that will not cause endotoxicity but will bind to antibody in already infected animals could be a way to deliver the complex to spleens. Outer membrane vesicles (OMVs) obtained from *B. melitensis* can be tested for this purpose. OMVs contain both outer membrane proteins as well as LPS that are the major antigens for antibody reactions in infected host. The risk associated with LPS endotoxicity should be considered first. To eliminate this risk OMVs from rough strain *B. abortus* RB51 could be used.

The next focus of this dissertation was to test the effect of immunostimulants on the clearance of *Brucella* from the infected host. Immunotherapy modulates the immune system and helps it to fight against infections or cancers (33). The concept is widely tested in cancer treatment and for the treatment of chronic infections (13, 33, 34). During chronic infection there is a switch from a Th1 to a Th2 type immune response in humans infected with *Brucella* (20). *Ex-vivo* stimulation of T-cells from these patients shows a lack of stimulation or blastogenesis upon treatment with mitogens or *Brucella* antigens (28). T-cell anergy and failed action against *Brucella* lead to the development of chronic infection. Thus, stimulating the immune system should help it to fight against *Brucella*. For the purpose of immunostimulation, we focused on testing immunostimulants that would provide specific stimulation against *Brucella*. Vaccination or immunization is based on the principle of immunostimulation by immunogens/vaccines (35). Thus vaccines are immunostimulants that provide

specific immunostimulation and could be used for immunotherapy (15). All approved vaccines for brucellosis are live-attenuated, making them potentially capable of causing infection (23). Thus, a subunit vaccine would be a better choice. Vaccination with irradiated *B. neotomae* has been shown to provide protection against all major species of *Brucella* that cause infection in human (17). Moreover, *B. neotomae* is not known to cause infection in humans or other animal species (31). Thus, irradiated *B. neotomae* was one option to be tested as an immunostimulant in a *Brucella* infected host. The second option was OMVs obtained from *B. melitensis*. Vaccination with *Brucella* OMVs has been shown before to provide protection against challenge with *B. melitensis* in mice (2). Being a subunit vaccine, the choice of adjuvant was critical to enhance the immunogenic efficacy of OMVs and was the focus of chapter 3. We tested Pluronic P85 as an adjuvant that has been shown before to enhance the efficacy of DNA vaccines (8, 11). Other pluronics like L-121, F69, CR-1024 have been shown to be effective adjuvants for subunit vaccines but because they have surfactant- like properties, they are found to be toxic at the site of injection (3). Because P85 is ampiphilic in nature, it is considered less toxic than hydrophobic pluronics (8). We have shown that P85 itself provides significant protection when used as a vaccine against *B. melitensis* challenge. OMVs mixed with P85 provided higher protection than OMVs alone but the mechanism could not be fully deduced. Higher IgG antibodies in the plasma against *B. melitensis* were measured in the mice vaccinated with OMVs+P85 compared to OMV vaccinated mice and could be the reason for higher protection. Although *Brucella* is an

intracellular pathogen, antibodies have been shown to provide protection against *B. melitensis* (6, 14). P85 vaccinated mice also showed significantly higher protection than saline vaccinated mice. The mechanism is still not clear but similar Pluronic activity has been shown before (19). Thus, the protection provided by OMVs+P85 could be the additive effect of OMVs and P85. Future work should focus on finding the mechanism by which Pluronics provide protection against bacterial challenges. Moreover, different *Brucella* protective antigens that are present in the outer membrane, when over expressed have been shown before to enhance the efficacy of the parent *Brucella* vaccine (21, 36). Thus OMVs from *Brucella* strains over-expressing these protective antigens (e.g. superoxide dismutase (SOD), Omp31, Omp25 etc) should be tested as vaccine. Additionally, rough mutants of *B. melitensis* should be generated to obtain OMVs without LPS. This would help with differentiating vaccinated vs. infected subjects on the basis of LPS antibodies.

The efficacy of immunostimulants/vaccines on the treatment of brucellosis was tested in chronically infected mice. Irradiated *B. neotomae* and OMVs with P85 as an adjuvant were tested as immunostimulant/vaccine candidate. Vaccines were tested alone or in combination with doxycycline-gentamicin antibiotic treatment. In this study, we deliberately used the lowest recommended dose of doxycycline to create a treatment failure at the end of the therapy. This was expected to help to evaluate the additive effect, if any, of antibiotics and vaccines on the clearance of *B. melitensis* from infected mice. There was no additive effect of vaccines and antibiotics on the clearance of *Brucella* from mice. Mice that

received vaccine along with antibiotics were able to prevent the multiplication of *Brucella* after the end of treatment. These combinations might be able to help in better management of *Brucella* cases with treatment failures or relapse. Irradiated *B. neotomae* was considered better than OMVs as the immunostimulant as it was not associated with any sickness in mice. With regards to stimulation with heat inactivated *B. melitensis*, splenocytes from DG+ IBN treated mice showed higher secretion of IFN-γ compared to splenocytes from DG treated mice. The mice treated with DG + OMVs showed higher levels of IgG antibodies against *Brucella* antigens compared to DG treated mice. IBN and OMVs generated stimulated the immune system differently but both strengthened the immune response in chronically infected mice against *Brucella.*

There are many opportunities that can be explored in the future. There is no report of generating a *Brucella* relapse animal model. There are several reports on *Mycobacterium* relapse mouse models that open the door for research focused on controlling these relapses (4, 16). IBN or OMVs could be able to prevent relapses and should be tested for the same in the treatment of chronically infected brucellosis patients.

As OMVs were found to cause some endotoxicity upon administration in infected mice, procedures that can eliminate LPS from OMVs should be tested to create a safer immunostimulant to treat brucellosis. Other subunit vaccines like purified *Brucella* protective antigens must be tested in infected animals to stimulate the immune system in chronic *Brucella* infections. Another interesting avenue will be to determine the long-term fate of *Brucella* in untreated mice vs. treated mice.

Using a bioluminescent *Brucella*, Splitter's lab has shown that if remained untreated, *Brucella* migrated to tail bones in mice after 7-8 months of infection (22). Effect of treatment and immunostimulation on the localization of *Brucella* in different organs should be studied. This will help in understanding the migration of *Brucella* in infected hosts that allows it to escape killing by antibiotics and cause relapses.

From the present work we can conclude that:

1) Gentamicin loaded nanoparticles are very effective for cellular uptake and killing *Brucella in vitro.* However, when compared to free drug, these complexes fail to provide better clearance of *Brucella* in mice,
2) P85 enhances the efficacy of OMVs as a vaccine and by itself provides protection against *B. melitensis* challenge in mice through unknown mechanisms,
3) Combining immunostimulants with conventional antibiotics helps restrict the further multiplication of *Brucella* in cases of treatment failure.

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Addendum 1

Effect of erythritol on growth and survival of

Brucella in vitro and *in vivo*

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Abstract

Erythritol has been considered as a very important factor for the pathogenesis of *Brucella abortus* 2308 and its ability to cause abortion in ruminants. There is a lack of laboratory models to study the *Brucella*-erythritol relationship, as commonly used murine models do not have erythritol. We tested the effect of erythritol on the growth of *Brucella* in iron minimal medium (IMM), macrophage culture and in mice to determine if these laboratory models can be used to study the relationship between *Brucella* and erythritol. An effect of erythritol on *Brucella* growth was only seen in IMM. There appear to be no effect of erythritol on *Brucella* growth in macrophage cell culture or in mice. This shows that administration of erythritol to the mice cannot mimic the environment in ruminants during pregnancy and thus cannot be used as a model to understand the effect of erythritol on *Brucella* pathogenesis.

Introduction

One of the major breakthroughs in *Brucella* research came when one of the chemical basis of *Brucella* pathogenesis was determined to be erythritol [14]. Erythritol, a four-carbon alcohol sugar, is found in small amount in some fruits and in very high concentrations in fetal tissues in ruminants. Mammals are unable to metabolize erythritol as carbon source. Some microorganisms have the ability to metabolize erythritol [6]. *Brucella* can metabolize erythritol and prefers it over other sources of carbon [20]. It has been shown before that erythritol promotes the growth of *Brucella* in enriched culture medium [17]. The ability of *Brucella* to cause abortions in ruminants has been associated with the presence of erythritol in trophoblasts and fetal tissues [19]. It is believed that rapid multiplication of *Brucella* in erythritol rich trophoblasts leads to the lesions in placenta and finally to the abortion [9]. Vaccine strain *B. abortus* S19 has partially deleted *eryC* and *eryD* genes (involved in erythritol metabolism) and shows inhibition of growth in presence of erythritol in culture medium [15]. This was thought to be the reason for its inability to cause abortions in pregnant ruminants. However, when same gene deletions were generated in wild type *B. abortus*, the mutants were not attenuated in peritoneal murine macrophages and in mice compared to wild type strain [12]. Thus, it was considered that additional factors were involved in the attenuation of S19. However, the pathogenesis of these mutants compared to wild type strain and S19 were never studied in pregnant ruminants. Other *Brucella* mutants that showed growth attenuation in the presence of erythritol in IMM included *entC* and *entF* (genes involved in siderophore biosynthesis and iron acquisition) deletion mutants [1, 5]. Although, compared to the wild type strain, the *entC* deletion mutant did not show any difference in pathogenesis in mice [2]. It failed to cause abortions in pregnant ruminants like wild

type strain [3]. It was not possible to test *entF* deletion mutant in pregnant ruminants due to expenses and intensive labor required.

Laboratory mouse models are widely used and accepted in *Brucella* research [13]. However, erythritol is not as abundant in rodents as it is in ruminants; therefore, rodents are not appropriate to study *Brucella*-erythritol relationship [13]. No information has been published on artificially injecting erythritol in *Brucella* infected mice and determining if it affects *Brucella* growth. In the present work we aimed to determine the effect of erythritol on survival and growth of wild type *B. abortus* in iron minimal medium, macrophage culture and in mice and to determine if exogenous addition of erythritol can be used to develop laboratory models to study the *Brucella*-erythritol relationship.

Material and methods

<u>Bacterial strains, cell lines, mice strain</u>: Wild type *B. abortus* 2308 (our stock culture, Virginia Tech.) was used. J774A.1 murine macrophages like cells were used for *in vitro* studies. J774A.1 cells were regularly grown in Dulbeco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Approximately, 6-8 weeks old female BALB/c mice (Harlan Laboratories, USA) were used for *in vivo* experiments.

<u>Growth in iron minimal media:</u> Iron minimal media was prepared as previously described [7]. The amount of iron in IMM was measured using atomic absorption spectrophotometry (flame method) and was found to be ≤ 0.998 μg/mL. Cultures in IMM started at 10^6 CFU/mL from the frozen stock culture of *B. abortus* 2308. Erythritol was

added to the IMM before starting the cultures. To monitor the growth at different time points, 200 µL of culture was sampled in triplicates and 10 fold serial dilutions were made in PBS and 50 µL of each dilution was plated on TSA plates to determine *Brucella* CFUs after 48-72 hr of incubation at 37°C and 5% CO_2.

<u>Survival and growth in macrophages</u>: The effect of erythritol on *in vitro* survival and replication of *B. abortus* was tested in J774A.1 macrophages. Macrophages were seeded at a density of 5 x 10^6 cells/well in a 24-well cell culture plate (Corning Inc.) 24-36 hr prior to infection. At 90% confluency in the wells, the cells were infected with *B. abortus* 2308 for 1 h at a multiplicity of infection of 1:100. After phagocytosis, the media was removed and fresh media containing 50 µg/mL gentamicin was added and the cells were incubated for 45 min to kill extracellular bacteria. The media was removed and the infected cells were incubated with DMEM+10% FBS for 24 hr to set up the infection model. At 24 hr post-infection, the cells were washed twice with DMEM and 2 mL fresh medium with and without 0.25% erythritol added to the cells and cells were incubated again. After 2, 4, 8, 12 and 24 hr of incubation the media was removed and the cells were washed twice with PBS. To determine the intracellular bacterial load, the cells were lysed using 250 µL of 0.1% Triton X-100™ and 10-fold serial dilutions of lysates were prepared and spread on TSA plates. Colony forming units (CFUs) were determined after incubating the plates for 48 hr at 37 °C under 5% CO_2

<u>Survival and replication of *Brucella* in mice:</u> First we determined the LD_{50} of erythritol in *Brucella* infected mice. A total of 15 BALB/c mice were infected with *B. abortus* 2308, 5 x 10^4 CFUs injected i.p. After 6 weeks of infection, mice were divided in three groups

and injected with erythritol at concentrations 2, 4 and 8 gm/kg body weight and were monitored for 4 days for signs to any toxicity. Later we determined the effect of single or multiple doses of erythritol on *Brucella* survival and growth in mice. A total of 20 BALB/c mice were infected as before and after 6 weeks of infection mice were divided into four groups. Groups of mice were either injected with saline or with 1,2 or 3 doses of erythritol (4 gm/kg body weight) 4 days apart. Mice were euthanized after 24 hr of last dose and *Brucella* CFUs were determined in spleens and livers after homogenizing the organs and culturing the lysates on TSA plates.

Results

Effect of erythritol of *Brucella* growth in iron minimal medium: Growth of *B. abortus* 2308 slowed down in IMM supplemented with 0.1% erythritol (Figure A.1.1) compared to growth in IMM. In the presence of erythritol, *Brucella* appeared to be surviving but not growing. This effect was reversed with the addition of $FeCl_3$ at a concentration of 50 μg/mL. This shows that iron was the limiting factor. Growth in IMM was also improved with the addition of $FeCl_3$ and significantly higher growth was seen in IMM with erythritol and $FeCl_3$ both compared to IMM + $FeCl_3$. Thus, the growth pattern of *Brucella* in IMM and IMM + supplements was in the following order: IMM + erythritol < IMM < IMM + $FeCl_3$ < IMM + erythritol + $FeCl_3$.

Intracellular survival and growth of *Brucella* in macrophages: Murine J774A.1 macrophage like cells were infected with *Brucella* and were allowed to develop an infection before addition of erythritol to the medium to study the differences in survival and growth of *Brucella*. No differences were observed in intracellular *Brucella* CFUs in

the cultures with or without erythritol (Figure A.1.2). Further, addition of the iron chelator, deferroxime mesylate (DFA) and erythritol together did not affect the survival and growth (data not shown).

<u>Effect of administration of erythritol in *Brucella* infected mice:</u> Mice were infected with *B. abortus* and were allowed to develop a chronic infection, characterized by persistence of *Brucella* in spleens and livers [8], before injecting erythritol. Initially we tested the appropriate concentration of erythritol that mice would tolerate without having any ill effects. Mice were given 2, 4 or 8 gm/kg body wt. erythritol. Injecting 8 gm/kg body wt. erythritol caused 60% (3/5 mice) death in mice within 12 hr of injection. All the mice showed signs of discomfort including dehydration and shivering. Mice that received either 2 or 4 gm/kg body wt. erythritol did not show any signs of discomfort. For further mice experiments, erythritol was used at the concentration of 4 gm/kg body wt. per mouse. Mice were given either 3 doses of erythritol (on 42nd, 46th and 50th day post infection), 2 doses (on 46th and 50th day post infection) or single dose (50th day of infection) of erythritol. No effect of *Brucella* CFUs in spleen or liver was seen with the administration of erythritol (Figure A.1.3).

Discussion

A link between *B. abortus* pathogenesis and erythritol was established almost 50 years ago but it is still not clearly understood. One of the major reasons for this is the lack of laboratory models to study *Brucella*-erythritol relationship. Further, the relationship between erythritol metabolism and iron availability in *B. abortus* has been established

but not fully understood [11]. Iron minimal medium (IMM) supplemented with erythritol has been shown as an initial screening tool to study mutants deficient in iron acquisition [1, 5]. It has been shown before that erythritol enhances the growth of *Brucella* in enriched media [17]. Figure A.1.1 shows that addition of erythritol to IMM reduces the growth of wild type *B. abortus* when compared to the growth in IMM without erythritol. However, this growth attenuation was reversed and *Brucella* showed an enhanced growth in IMM supplemented with both erythritol and iron. Thus, it can be concluded that erythritol enhances *Brucella* growth but essentially requires the presence of iron. IMM supplemented with erythritol can be useful to study *Brucella* mutants involved in erythritol metabolism and iron acquisition.

Further, we tested the effect of addition of erythritol on intracellular survival and growth of *Brucella* in macrophages. Theoretically, if utilization erythritol enhances the replication of *Brucella* then macrophage culture medium supplemented with erythritol should enhance replication of intracellular *Brucella*. Macrophages are naturally iron-restricted environment and have minimal iron. Upon infection macrophages reduce their transferrin receptors under the influence of IFN-γ and uptake less iron [18]. Moreover, natural resistance associated macrophage proteins (Nramp1 and Nramp2) further reduce the iron inside phagosomes and late endosomes by mediating the active efflux from the site [4, 21]. Thus, macrophage represents iron-restricted environment and *Brucella* growth inside macrophages should be impeded in the presence of erythritol. Contrary to both the theoretical possibilities, in the present study we showed that addition of erythritol to the culture medium does not affect the survival and growth of *B. abortus* 2308 in J774A.1 cells. Further, addition of iron-chelating agents to limit the iron

failed to affect the intracellular growth of *Brucella*. Thus, macrophages cultured in medium containing erythritol cannot be used as the *in vitro* model to study effect of erythritol on *Brucella*.

Mice models have been widely used for *Brucella* research [13]. Different *Brucella* gene deletion mutants involved in erythritol metabolism and siderophore biosynthesis have been tested in mice but did not show differences in pathogenesis from wild type strain [2, 12]. Rodents do not have erythritol and, thus, are not good models for testing the attenuation of mutants with respect to ability to cause abortions. However, it has not been studied before if administration of erythritol affects *Brucella* in mice. In our study we found that administration of 8 gm/kg body wt. erythritol caused 60% mortality in mice within 4 hr of injection. The remaining mice died within 72 hr of injection. Previous reports have shown the LD_{50} of erythritol in rodents is 9.6 gm/kg body wt when administered intravenously (i.v.) [10]. Mice have been shown to tolerate erythritol up to 45 gm/kg body wt when given mixed with food without any toxicity [16]. This difference may be due to the difference in the strain of mice or may be because in our study mice were infected with *Brucella*. All of the dead mice were found to have excessive fluid in the peritoneal cavity. Erythritol may have caused osmotic imbalance leading to death of the mice. Chronically *Brucella*-infected mice treated with single, double and triple doses of 4 gm/kg body wt. of erythritol showed no differences compared to non-treated mice in the *Brucella* CFUs in spleens or livers. This might be due to the rapid elimination of erythritol from the mice or achieved serum concentrations were not high enough. Moreover, erythritol might not be reaching the organs where *Brucella* is present in chronically infected mice.

In conclusion we have shown that erythritol affects the *Brucella* growth only in IMM but had no effect on *Brucella* growth in macrophages or in mice. Thus, neither the macrophage cell line nor the BALB/c mice are suitable models for the study of erythritol mechanism related to *Brucella* pathogenesis.

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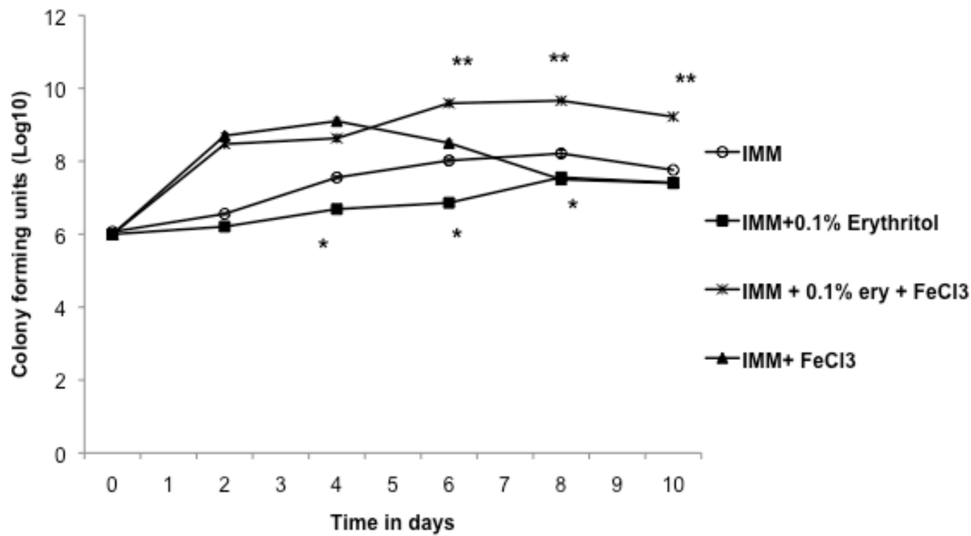

Figure A.1.1: Growth of *B. abortus* 2308 in iron minimal media compared to IMM supplemented with 0.1% erythritol or 50µM $FeCl_3$ or both.

All the cultures were started with $1x10^6$ CFUs/mL and 200 µL sample was taken at every time point in duplicates to determine the CFUs. Addition of erythritol significantly attenuates the growth of *B. abortus* 2308 while addition of $FeCl_3$ reverses the effect of erythritol. Data presented here is the mean ± SEM of two independent experiments. One asterisk (*) and two asterisks (**) represent the statistically significant ($p \leq 0.001$) difference from the growth in IMM and IMM+$FeCl_3$ respectively.

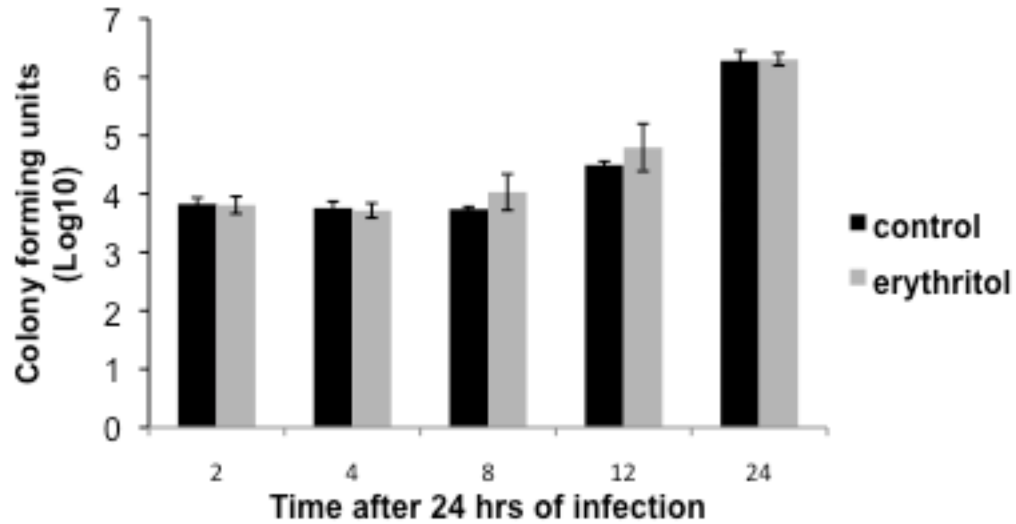

Figure A.1.2: Effect of erythritol on intracellular growth and survival of *B. abortus* 2308 in J774A.1 macrophages.

Macrophages were infected with *B. abortus* 2308 at an MOI 1:100 and allowed to establish an infection. After 24 hr of infection, cell were washed with PBS and incubated either with macrophage culture medium or medium supplemented with 0.25% erythritol. At each time point cells were washed and lysed using TritonX and intracellular *Brucella* CFUs were determined. The data represents mean ± SEM of three independent experiments.

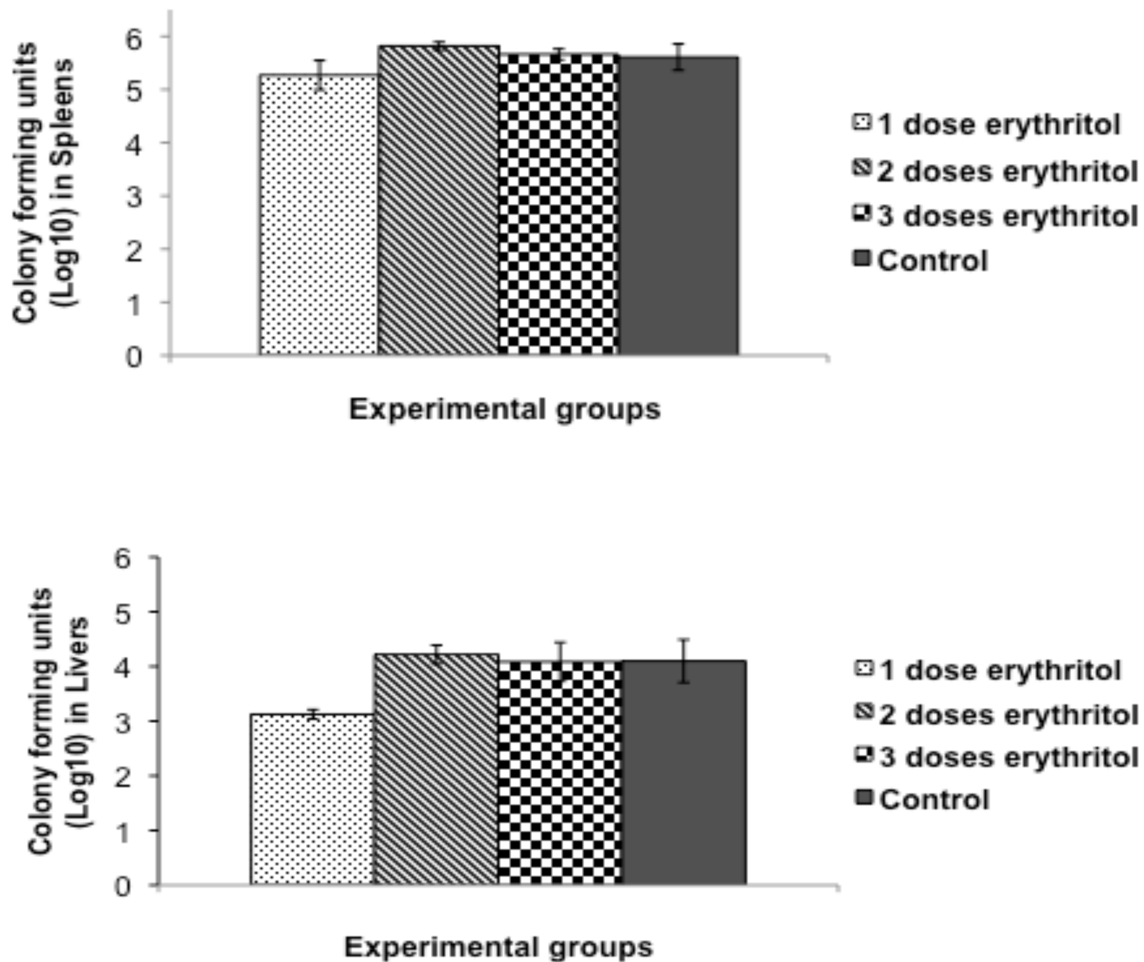

Figure A.1.3: Effect of single or multiple dosing of erythritol on *Brucella* counts in spleens and livers of *Brucella* infected mice.

Mice were infected with *B. abortus* 2308, after 6 weeks of infection mice were injected 4 gm/kg body weight erythritol (i.p.). Single, double or triple doses of erythritol were given. Saline administered mice were control group. Mice (n=5) were euthanized 24 hr after the last dose and *Brucella* CFUs were determined in spleens and livers. Top and bottom panels show the *Brucella* CFUs in spleens and livers respectively. The data represents the mean ± SEM from five mice in each group.

Addendum 2

Combined effect of gentamicin loaded magnetite block ionomer complexes (MBICs) and immunostimulants on *Brucella melitensis* clearance in mice

Chronic infections like tuberculosis and brucellosis are very difficult to treat due to the lack of supporting host immune response against these pathogens (2, 3) and intracellular localization of the pathogen, which is hard to be reached by antibiotics (1). In chapter 4, we have shown that combining antibiotics with vaccines prevents the multiplication of remaining *Brucella* in the host after the end of treatment. In the present study, we hypothesize that this effect can be enhanced with the use of gentamicin loaded MBICs. In chapter 2.2 we showed that MBICs did not show higher efficacy compared to free gentamicin to eliminate *Brucella* from infected mice in combination with doxycycline. But histopathological examination of kidneys from MBICs treated mice showed the presence of brown pigment laden macrophages in peri-renal adipose. After staining the kidney sections with Perl's Prussian blue, the brown pigment was found to be iron and must be the magnetite core of MBICs. We hypothesize that these deposited MBICs might be able to release drugs during their clearance from host and might show a different effect than free gentamicin after the end of the treatment. In chapter 4 we have shown the potential of outer membrane vesicle (OMVs) and irradiated *Brucella neotomae* (IBN) as immunostimulants to

treat *B. melitensis* infection in mice in combination with antibiotics. Although there was no additive effect of antibiotics and immunostimulants on the clearance of *Brucella* from infected mice, mice that received immunostimulants prevented the replication of *Brucella* after the treatment failure in the mice. In the present study we used OMVs from *B. melitensis* rough mutant *B. melitensis* VTRM1 (4). As MBICs were synthesized and characterized as described in chapter 2.2. OMVs were obtained from *B. melitensis* VTRM1 following the same procedure as described in chapter 3. The mice were infected and treated in the same way as described in chapter 4.

OMVs obtained from VTRM1 also induced mild sickness in *Brucella* infected mice as shown by the OMVs from smooth strain. Table A.2.1 shows the *Brucella* CFUs in spleens and livers 4 weeks after the end of the treatment. MBICs treated mice showed similar *Brucella* loads in spleens and livers as free gentamicin treated mice in combination with doxycycline. Moreover, combining the immunostimulants with antibiotics resulted in similar *Brucella* CFUs in mice irrespective of the choice of source of antibiotics (free or MBICs) and immunostimulant.

Thus, we can conclude that combining immunostimulants with antibiotics might help in better management of *Brucella* infected cases after a treatment failure and might be able to prevent relapses in completely treated cases. But MBICs did not enhance the clearance of *Brucella* as well as its control after treatment failure.

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Table A.2. 1: Comparative efficacy of MBICs and free gentamicin in combination with doxycycline and immunostimulants to control *Brucella* replication after treatment failure in mice.

Treatment	Immunostimulant	CFUs in Spleens (Log_{10})	CFUs in Livers (Log_{10})
Saline	Saline	3.19 ± 0.42	2.29 ± 0.49
Doxy-Genta	Saline	5.18 ± 1.00	3.26 ± 1.17
Doxy-Genta	OMVs (VTRM1)	2.88 ± 0.93**	2.38 ± 1.87**
Doxy-Genta	IBN	2.09 ± 0.43**	1.6 ± 0.00**
Doxy-MBICs	Saline	4.17 ± 1.03	2.33 ± 1.46
Doxy-MBICs	OMVs (VTRM1)	2.28 ± 0.88**	1.6 ± 0.00**
Doxy-MBICs	IBN	1.6 ± 0.00**	1.6 ± 0.00**

Mice were infected with *B. melitensis* 16M and treated after 7 weeks of infection. Gentamicin, either free or loaded in MBICs, was given in combination with doxycycline for 28 days. Immunostimulant irradiated *B. neotomae (*IBN) or outer membrane vesicles (OMVs) obtained from *B. melitensis* VTRM1 were given twice during the treatment. Saline treated mice were the negative controls. Mice were euthanized 4 weeks after the end of the treatment and *Brucella* CFUs were determined in spleens and livers. The data represents mean ± SEM (n=4) and double asterisk (**) represents statistically significant difference from the mice treated with saline and from antibiotics alone.